Prevention and Control of Lower Airway Inflammation in Horses

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April 2008

RIRDC Publication No 08/051
RIRDC Project No US-118A
Foreword

Lower airway inflammation (LAI) is highly prevalent in young performance horses resulting in detriments to respiratory health and performance. As a result, the control and prevention of this syndrome is critical to performance horse industries, particularly the Thoroughbred industry. Studies in Australia and elsewhere indicate that LAI, and the associated accumulation of tracheal mucus, are common findings occurring in up to 50% of racehorses in training. The consequences of LAI include decreased performance, lost training days, and costs of treatment, all of which result in substantial economic losses for the industry.

Treatment and management of LAI in Australia has been largely empirical to date, and based on anecdotal evidence, or studies of respiratory tract disease in older horses with more chronic respiratory disease. The aim of the current study was therefore to critically assess the efficacy of these treatment regimens for resolution of airway inflammation in young, performance horses housed intensively under Australian conditions. Additionally, the study was designed to review the contributions of various environmental sources for known airway irritants, thereby providing information on both management and pharmaceutical intervention for this syndrome.

These studies will be of great benefit to performance horse industries by providing up to date information regarding strategies for the prevention and control of LAI. The studies will also determine how to better address the highly prevalent problem of coughing horses with poor performance and excess tracheal mucus. Results have significant practical application, creating stable management recommendations and inhalational therapy guidelines for the prevention and control of LAI.

This project was funded by the Rural Industries Research and Development Corporation (RIRDC), with matched funds provided by the Australian Government. Additional financial support was also received from The Waltham Foundation (United Kingdom) and The Thoroughbred Racing Board of NSW and The University of Sydney.

This report, a new addition to RIRDC’s diverse range of over 1800 research publications, forms part of our Horse 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:

- purchases at www.rirdc.gov.au/eshop

Peter O’Brien
Managing Director
Rural Industries Research and Development Corporation
Acknowledgments

The authors gratefully acknowledge the following:

The Rural Industries Research and Development Corporation (Horse R&D), The Waltham Foundation (United Kingdom), The University of Sydney and The Thoroughbred Racing Board of NSW for their financial support, which was integral to the successful completion of the experimental studies contained herein.

We also acknowledge the invaluable contributions made by Dr Nick Malikides (Novartis Animal Health), Greg Hogan (University of Sydney Horse Unit), Maryann O’Donnell (Statistical Advisory and Training Services) and Nick Basgallop (NSW Racing Forensic Laboratories). Furthermore, scientific collaborations with Professor Stuart Reid and Dr Dominic Mellor of The University of Glasgow were paramount to the planning and success of each of the experiments reported herein.

Lastly, sincere thanks and appreciation goes to the Thoroughbred racehorse trainers from Warwick Farm, Rosehill, Hawkesbury and Camden whose eagerness and willingness to participate in research was integral to the success of the investigations.

All the experiments were approved by the University of Sydney’s Animal Care and Ethics Committee.
Abbreviations

ANOVA     Analysis of variance
APCI      Atmospheric pressure chemical ionisation
Av        Average
BAL       Bronchoalveolar lavage
CFC       Chloroflurocarbon
cfu       Colony forming units
cm        Centimetre
CRD       Completely Randomised Design
EIPH      Exercise induced pulmonary haemorrhage
EU        Endotoxin unit
g         Grams
HBA       Horse blood agar
HFA       Hydrofluroalkane
HPLC      High performance liquid chromatography
hrs       Hours
IAD       Inflammatory airway disease
IOM       Institute of Occupational Medicine
IV        Intravenous
JN        Jet nebuliser
kg        Kilogram
km        Kilometre
KOH       Potassium hydroxide
L         Litre
LAI       Lower airway inflammation
LAL       Limulus amoebocyte lysate
LCMS      Liquid chromatography mass spectrometry
LOD       Limit of detection
Log_e     Natural logarithm
LPS       Lipopolysaccharide
LRT       Lower respiratory tract
LRTI      Lower respiratory tract inflammation
m²        Metre squared
m³        Metre cubed
MDI       Metered dose inhaler
μL        Microlitre
μm        Micrometre
min       Minutes
mL        Millilitre
mol/L     Moles per litre
ND        Nasal discharge
ng        Nanogram
ng/m³     Nanogram per cubic metre
ng/mg     Nanogram per milligram
nm        Nanometre
NSAID’s   Non steroidal anti inflammatory drugs
PAM       Pulmonary alveolar macrophage
PAS       Personal air samplers
pg         Picograms
OM        Organic matter
RAO       Recurrent airway obstruction
RCT       Randomised Clinical Trial
RE        Respirable endotoxin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SB</td>
<td>Standardbred</td>
</tr>
<tr>
<td>SRM</td>
<td>Selective reaction monitoring</td>
</tr>
<tr>
<td>TA</td>
<td>Tracheal aspirate</td>
</tr>
<tr>
<td>TAF</td>
<td>Tracheal aspirate fluid</td>
</tr>
<tr>
<td>TB</td>
<td>Thoroughbred</td>
</tr>
<tr>
<td>TDG</td>
<td>Tracheal discharge grade</td>
</tr>
<tr>
<td>TMR</td>
<td>Total mixed ration</td>
</tr>
<tr>
<td>TLV</td>
<td>Threshold limiting value</td>
</tr>
<tr>
<td>UN</td>
<td>Ultrasonic nebuliser</td>
</tr>
<tr>
<td>URT</td>
<td>Upper respiratory tract</td>
</tr>
<tr>
<td>UTS</td>
<td>Unbalanced Treatment Structure</td>
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Executive Summary

What the report is about
This report delivers the findings of a study to assess the efficacy of inhalational pharmaceutical treatment options for treatment of lower airway inflammation (LAI) in young performance horses housed intensively in stables, and additionally to review the contributions of various environmental sources for known airway irritants. Information on both management and pharmaceutical intervention for this syndrome is provided.

Who is the report targeted at?
Study results will be of great benefit to the performance horse industries by providing up to date information regarding strategies for the prevention and control of LAI and how to better address the highly prevalent problem of coughing horses exhibiting poor performance and excess tracheal mucus. Results have significant practical application, creating stable management recommendations and inhalational therapy guidelines for the prevention and control of LAI.

Background
Lower airway disorders are among the most common conditions encountered by equine veterinarians and are reported to be second only to musculoskeletal disease as a cause of wastage among performance horses (Rossdale, Hopes et al. 1985), with up to 33% of young racehorses in training in Australia demonstrating lower airway inflammation in the absence of overt clinical signs of respiratory disease (Malikides and Hodgson 2003). In 1996, the Rural Industries Research and Development Corporation (RIRDC) conducted an extensive research questionnaire into the needs of the Australian horse industry. This survey revealed that two of the most pertinent issues relating to horse health were evaluation and treatment of respiratory disease and determination of the causes and treatment of poor performance.

A number of recent studies have shed light on the risk factors for development of Lower airway inflammation (LAI) in young racehorses in Australia, in particular environmental agents that may induce airway inflammation (Christley, Hodgson et al. 2001; Malikides 2003). These studies have enabled an increased focus on these agents, but little is known about management practices that may impact on their presence/concentrations in the environments in which horses are housed. In addition, primary reliance for treatment of airway inflammation in Australian performance horses has been based on systemically or orally administered therapeutic agents, which have been used to treat chronic airway disease in older horses. Little information is available regarding the use and efficacy of airway specific, aerosolised medications in young, performance animals.

Objectives
The aims of the current research were to assess the efficacy of inhalational pharmaceutical treatment options for treatment of lower airway inflammation in young, performance horses housed intensively in stables, and additionally to review the contributions of various environmental sources for known airway irritants, thereby providing information on both management and pharmaceutical intervention for this syndrome.

Results Summary
Experiment 1: Endotoxin Studies
Endotoxin is a recognised cause of lower airway inflammation in both humans and horses. However, specific sources of endotoxin have not been identified within equine environments. This study identified bedding type to be a significant factor (p=0.001) in contributing to aerosolised stable endotoxin concentrations, and different bedding types generated difference concentrations of endotoxin in the horse’s breathing zone. In contrast, no difference was detected for total and respirable particles, and particle endotoxin exposures generated by the four different feed types
examined. Thus, all feed types in this study produced a similar airborne endotoxin concentration in the breathing zone of horses, and feed was not a significant contributor to overall stable load.

The significant differences observed in this experiment for total and respirable endotoxin concentrations between bedding types may be interpreted to suggest that appropriate selection of bedding type may provide an effective method for lowering airborne endotoxin concentrations within the breathing zone of stabled horses. In contrast, results from this study would suggest that changing feed type may be a less effective method for decreasing endotoxin concentrations within a stable.

**Experiment 2: Inhalational Therapy Pilot Study**

A small pilot study was conducted to assess the efficacy of aerosolized agents, used for treatment of chronic LAI in older horses, in a cohort of younger horses. These results were also used to inform decisions regarding the choice of therapeutic agents and length of time of treatment in the larger, randomised clinical trial (Experiment 4).

Horses with airway inflammation (>20% neutrophils in tracheal aspirates) were included in the study and received one of three treatments (a corticosteroid, fluticasone propionate; a bronchodilator, ipratropium bromide; or a combination of these two drugs). A fourth group of horses received a placebo control. Results suggest that treatments were not equally efficacious for decreasing pulmonary neutrophilia, where a significant difference between treatment efficacy (p = 0.044) over the course of therapy (14 days) was found. At the conclusion of the study, horses receiving the control (placebo) demonstrated an increased percentage of neutrophils (+14.2%), compared to those treated with the combination (-18.5% neutrophils) or fluticasone propionate alone (-22.0% neutrophils), the latter group having a significant decrease in percentage of neutrophils (p = 0.012) as compared to control horses. In contrast, there was found to be no statistically significant difference between control horses and those receiving the bronchodilator alone (p = 0.82), indicating the use of a bronchodilator for the treatment of airway inflammation is ineffectual and therefore this treatment was not recommended for inclusion in the Randomised Clinical Trial (RCT). However, as the combination of a corticosteroid and a bronchodilator approached significance in this small study, the combination therapy was also included in the RCT.

Changes in neutrophil percentages over the course of the study were also evaluated, where there was a highly significant difference for treatments between Day 0 and Day 7 (p = 0.003), but no significant difference for treatments between Days 7 and 14. However, it was felt this result reflected the decrease in proportions of neutrophils in control horses over this time period and was not an indication of ongoing efficacy of the therapies investigated. Hence, it was recommended that a second week of therapy should be included in the RCT to further tease out the relationship between time of treatment and efficacy.

**Experiment 3A/3B: Inhaled Medication Drug Detection Studies**

The Australian Rules of Racing (A.R.178B) state that inhaled pharmaceutical agents, including ipratropium bromide (Atrovent®) and fluticasone propionate (Flixtotide®), are prohibited substances and cannot be detected in samples obtained from horses on race day. However, these agents are commonly used to treat lower respiratory tract disease in horses, including performance horses. Currently, there is a paucity of data on drug elimination studies for these aerosolised inhalational preparations that could be used to guide veterinarians and trainers in the appropriate use of these therapeutic agents.

Six standardbred horses were administered standard doses of these inhalational therapeutic agents for three days. At the end of drug administration, urine samples were collected by catheterisation at 0, 3, 6, 9, 12, 24, 48, 72 hours. All samples were analysed using Australian standard methodology at the Australian Racing Forensic Laboratory.
Results showed that large variations in the metabolism of ipratropium bromide and fluticasone propionate exist between horses. In general, ipratropium bromide was detectable up to 72 hours post administration with peak excretion at three hours post cessation of treatment followed by a rapid decline in excretion between six and nine hours post-administration.

Metabolites of fluticasone propionate could also be detected in equine urine after cessation of administration, where the limit of detection (LOD) for positive confirmatory purposes as detected by liquid chromatography mass spectrometry (LCMS) was 5 ng/mL, with a concentration of 500 pg/mL being detected by preliminary screening purposes. No metabolites of fluticasone propionate could be detected nine hours after cessation of treatment in the six horses included in this study.

However, for both drugs investigated, it should be remembered that significant inter-horse variability in metabolism, and therefore excretion, of the drug was observed. Therefore, the time at which samples were assessed to have negative detection of these drugs in the current experiment should not be extrapolated to the wider horse population, nor should the period of detection stated be interpreted as a recommended withholding period.

**Implications**

In conclusion, this study provides useful information for veterinarians treating performance horses with aerosolised therapy for lower respiratory tract disease. However, it must be remembered that the Australian Rules of Racing (A.R.178E) prohibit the administration of any medicinal substance to horses on race days. Therefore, the period of detection stated should not be interpreted as a recommended withholding period.

**Experiment 4: RCT for Evaluation of Efficacy of Inhaled Pharmaceutical Agents**

To date, regimes for treatment of LAI in young performance horses have been largely based on empirical therapy, using protocols derived for older horses with chronic small airway disease. Few field based clinical trials have been conducted on this population of horses to evaluate the efficacy of these pharmaceutical agents for reduction of LAI. Furthermore, the efficacy of these agents delivered via an inhalation route has not been evaluated in a large, clinical trial. The RCT conducted in this study was designed to determine if administration via inhalation of therapeutic doses of the corticosteroid fluticasone propionate, alone and in combination with the bronchodilator ipratropium bromide, provided therapeutic benefit in the absence of environmental control measures for the resolution of cough, pulmonary neutrophilia and increased tracheobronchial mucus in young performance horses.

Results from this study showed that there was no significant difference between treatments (fluticasone alone or in combination with ipratropium bromide) (p = 0.074) in resolution of pulmonary neutrophilia, but there was a highly significant difference between Days (Day 7-0, p=0.001; Day 14-7, p=0.001) for both treatments. In addition, no significant interaction was determined between Treatment and Day for neutrophil percentages (p=0.675; p=0.694), indicating that the response to each of the treatments was similar across each of the measurement times.

**Implications**

We conclude that aerosolised steroidal therapy is an effective and non-invasive way of treating LAI. From these results it seems logical to recommend that inhalation therapy should continue for 14 days if optimal treatment benefit is to be achieved. Further, the addition of a bronchodilator did not augment the overall anti-inflammatory effect and as such is probably not necessary for treatment of LAI. The use of a steroid alone would be less labour intensive, more cost effective, and hence, more likely to be applied in the field than combination therapy.
Review of Literature

Introduction
In recent years it has become increasingly apparent that lower airway inflammation (LAI) is common in Australian performance horses, particularly young Thoroughbreds (Christley 1999; Malikides 2003). More importantly, it has been recognised that this inflammatory process has the potential to impact upon the ability of these horses to achieve peak levels of performance. The athletic prowess of performance horses demands efficient function of the respiratory system, most importantly optimal gas exchange, which under normal conditions occurs at a minimal energetic cost to the animal (Robinson 1999). However, the alteration in respiratory tract function associated with inflammation and tracheal mucus accumulation may be sufficient to affect performance due to exacerbated hypoxaemia developing during exercise (Coutiel and De Nicola 1998). Furthermore, poor performance associated with airway inflammation results in lost training days (Jeffcott, Rossdale et al. 1982; Bailey 1998; Christley 1999), an inability to train horses effectively (Jeffcott, Rossdale et al. 1982; Bailey 1998; Christley 1999), incurs substantial losses due to costs of veterinary care and decreased earning potential (Malikides and Hodgson 2003) and finally, poses considerable welfare issues.

Lower airway disorders are among the most common conditions encountered by equine veterinarians and are reported to be second only to musculoskeletal disease as a cause of wastage among performance horses (Rossdale, Hopes et al. 1985), with up to 33% of young racehorses in training in Australia demonstrating lower airway inflammation in the absence of overt clinical signs of respiratory disease (Malikides and Hodgson 2003). A number of recent studies have shed light on the risk factors for development of LAI in young Australian racehorses, in particular environmental agents that may induce airway inflammation (Christley 1999; Malikides 2003). These studies have enabled an increased focus on these agents, but little is known about management practices that may impact on their presence in equine environments. In addition, primary reliance for treatment of airway inflammation in Australian performance horses has been based on systemically or orally administered therapeutic agents, with little information regarding the use and efficacy of airway specific, aerosolised medications.

The aim of this review is to briefly outline the structure and function of the equine respiratory system, with emphasis on non-specific defence mechanisms for prevention of airway inflammation. This will be followed by a discussion of lower airway inflammation in young performance horses including factors that have been identified associated with development of this disorder, clinical presentation and approach to diagnosis. Finally, the inhalational pharmaceutical treatment options, and their efficacy for the treatment of lower airway inflammation in young, performance horses housed intensively in stables will be reviewed in conjunction with environmental treatment and control measures.

The Equine Airway
The primary function of the respiratory system is to deliver oxygen to, and remove carbon dioxide from, the body and to accommodate the increased demands on the body for oxygen during exercise (Robinson 1986).

The adult equine respiratory tract is a series of branching tubules that allow large volumes of air to exchange, equating to approximately 1,800 litres/minute during intense exercise (Art, Anderson et al. 1989; Art and Lekeux 1993; Derksen and Robinson 2002). The respiratory tract therefore provides the largest interface between internal and external environments and for this reason, is consistently exposed to high levels of potentially deleterious air contaminants such as inorganic and organic particulate matter, along with airborne bacteria and other pollutants (Art, McGorum et al. 2002). In addition to delivering air to the gas exchange region of the lung, the tracheobronchial tree also protects this anatomical region from injurious inhaled agents and for this purpose, the tracheobronchial tree has developed a range of defence mechanisms (Derksen and Robinson 2001).
The galloping horse has less than half a second to inhale and exhale between 12 and 15 litres of air (Clarke; Clarke 1992), and hence are required to move large volumes of air efficiently to perform to their potential. For optimal gas exchange, approximately equal amounts of air and blood must reach each lung region, that is, ventilation and blood flow should ideally be matched (Robinson 1986). In cases of diseased lung tissue, regional variation in airway resistance and lung compliance results in uneven distribution of ventilation, with maldistribution of ventilation being a major cause of poor performance in horses.

**Deposition of Inhaled Particles**

The lung is the portal through which many gaseous and particulate irritants and allergens enter the body and the respiratory tract surface represents the body’s greatest area of contact with the environment (Yeates and Mortensen 2000). In addition, many particles act as vectors, carrying adsorbed pathogenic substances, such as endotoxin or micro-organisms, either in or on the particle surface, deep into the lower respiratory tract (Cargill 1999). Deposition of inhaled particles and their components within the airway can stimulate adverse effects. In horses, particle deposition most commonly triggers a non-specific inflammatory response which is dominated by neutrophil infiltration into the airways (Robinson, Derksen et al. 1986).

Traditionally, inhaled particles have been considered as being either respirable (i.e. capable of reaching the alveolar membrane) or non-respirable (i.e. those deposited in the upper respiratory tract or lower airways). Non-respirable particles are greater than 10-15µm in size and the majority of these are effectively removed by the vibrissae within the nares (Demeter, Pipoly et al. 1995).

**Pulmonary Defence Mechanisms**

Defence mechanisms of the respiratory membrane, both interdependent and coordinated, may provide adequate protection to a horse in a natural environment however, the defence mechanism becomes frequently overwhelmed by the stresses of domestication (Robinson 1999). Horses are routinely housed in confined spaces, which harbour a multitude of potentially injurious respirable particles. When interference with several defence mechanism occurs simultaneously (Bohning and Lipmann 1992), or when invading material (i.e. bacteria, particulate matter, endotoxin) is fittingly pathogenic and in adequate concentrations (Clarke 1987), or the foreign substance is unfamiliar to the host’s defence mechanisms (Demeter, Pipoly et al. 1995), inflammation invariably results.

**Mucociliary Clearance**

The mucociliary system is an integral part of the innate defence mechanism of the lung and plays a major role in the lung’s physiological defences against the myriad of inhaled particles the host must overcome daily. The mucociliary blanket extends from the pharynx to the level of the respiratory bronchioles, however, foreign particles may also be transported from the alveolar regions by various other mechanisms, proceeded by removal by mucociliary clearance. Particles deposited on the surface of the mucociliary system are transported out of the lung via the mucociliary escalator and swallowed, or alternatively, engulfed by macrophages or other cells recruited from the blood. In healthy humans, the mucociliary clearance mechanism can usually clear particles from the trachea within 6 hours. In healthy horses, the mean rate of mucociliary clearance is 2.1 ± 0.4 cm/min, with mucociliary transport rates being highly variable between horses but relatively constant in an individual.

The lower respiratory tract of healthy horses contains minimal or no muco-cellular material and low numbers of free cells (Hodgson 2005) however, as in various human respiratory pathologies, mucus accumulation in the horse is a characteristic, but non-specific, airway response associated with various infectious and environmental inflammatory airway diseases (Dixon, Railton et al. 1995). In the presence of disease, especially chronic conditions such as Recurrent Airway Obstruction (RAO), Inflammatory Airway Disease (IAD) and lower airway inflammation (LAI), mucus metaplasia of respiratory epithelium commonly occurs in the lower small airways (Kaup, Drommer et al. 1990) resulting in the accumulation of airway secretions. The amount of mucus present in the lower airways
increases with pulmonary irritation, specific causes of which may include bacteria, fungi or parasites, direct irritants or aeroallergens.

**The Cough Reflex**
Cough is a defence mechanism of the respiratory tract with two major functions, namely, prevention and limitation of inhaled foreign agents within the tracheobronchial tree and clearance of accumulated respiratory secretions in the airways. The sensitivity of the cough reflex is increased when the epithelium is damaged (i.e., by viral infections or inhaled particulate matter) (Robinson 1997).

Recent studies have investigated the usefulness of cough as a sign of lower airway disease in the horse, showing that cough is a highly specific, non-sensitive sign of lower airway disease (Burrell, Wood et al. 1996). In addition, cough is more common when airway disease has been present for more than one month, which explains why cough is found in a high proportion of horses (71%) referred for respiratory problems (Dixon, Railton et al. 1995). A number of studies have investigated coughing in young racehorses (Christley, Hodgson et al. 2001), where a series of four coughs or greater within a ten minute period was used to define coughing. These studies reported that 80-85% of coughing horses had evidence of lower airway inflammation and therefore coughing is a good positive predictor of lower airway inflammation, in young racehorses.

**Innate Cellular Pulmonary Defence Mechanisms; Neutrophils**
Neutrophils do not play a role in the primary defence against inhaled agents, but rather, are actively recruited into the lung tissue by chemo-attractants produced by bacteria (Ward, Lepow et al. 1968), macrophages (Hunningshake, Gadek et al. 1980) and neutrophils themselves (Wright and Gallin 1979). Under specific stimuli, such as particulate matter challenge or endotoxin inhalation, neutrophil adherence increases (Nourshagh 1993). Subsequently, neutrophils migrate into tracheal, bronchial and alveolar airways where they may quickly exceed macrophage numbers in airway secretions (See Figure 1).

Although a population of well-preserved neutrophils resides in horses airways, the relative proportion of these cells is considered to be normally low (Beech 1975; Mair, Stokes et al. 1987; Bain 1997; Hodgson 2005), comprising <20% of the nucleated cell population in the tracheobronchial tree in normal horses (Sweeney, Humber et al. 1992).

**Figure 1:** Smear of a tracheal aspirate (TA) demonstrating many neutrophils trapped within mucus strands. *(Diff Quik® stain; x1000)*.
Lower Airway Inflammation (LAI)

Lower airway inflammation is most commonly observed in Thoroughbred and Standardbred racehorses, but also has been reported in a variety of other breeds such as the Quarter Horse, Warmblood, Appaloosa and American Saddlebred (Vacher, von Fellenberg et al.; Macnamara, Bauer et al. 1990; Vrins, Doucet et al. 1991; Burrell, Wood et al. 1996; Couetil, Rosenthal et al. 2001).

Airway inflammation, as detected by either tracheal aspirate (TA) or bronchoalveolar lavage (BAL) occurs in 11.3% to 50% of Thoroughbred and Standardbred racehorses (Malikides 2003; Bayly 2005), with the duration of the disease having been variously described as ranging between 4 to 12 weeks, (Dixon, Railton et al. 1995; Dixon, Railton et al. 1995; Burrell, Wood et al. 1996). When increased quantities of mucopus visualised via endoscopy is used to define LAI, 22%-55% of Thoroughbred and Standardbred racehorses may be classified as having this disease (Burrell 1985; Macnamara, Bauer et al. 1990; Wood, Newton et al. 1999). Furthermore, the prevalence of LAI within a population, as defined by increased proportions of neutrophils (>20%) in TA samples, has been estimated to be 14% (Wood, Newton et al. 1999), 27% (Sweeney, Humber et al. 1992), and 33% (Burrell, Wood et al. 1996), with differences likely due to varied study design (i.e. longitudinal (Burrell, Wood et al. 1996) versus cross-sectional (Sweeney, Humber et al. 1992)) and differing populations of horses.

The disease appears to be more common in young athletic horses with the incidence decreasing with increasing age (Chapman and Green 2000; Christley, Hodgson et al. 2001). Interestingly, LAI is observed in performance horses worldwide and is not restricted to any particular country or geographical region, despite significant differences in management and climate (Robinson, Berney et al. 2003).

Risk Factors Associated with Cough & Airway Inflammation

Racehorses are a unique population of horses which differ from the general horse population in that they are predominantly young, with approximately 91% of a random sample of race starters in Sydney being aged between 2 and 5 years (Bailey, Reid et al. 1997), frequently participate in strenuous activity, are regularly transported, co-mingle with other horses (Burrell, Wood et al. 1996) and are predominantly housed in intensive stables (Rush Moore 1996; Christley 1999). All of these factors are associated with increased risk for the development of LAI in horses (Wood, Burrell et al. 1993).

More specific risk factors identified as being significantly associated with coughing include age (risk decreases with age) and training stage (horses in early training are at greater risk) (Christley, Hodgson et al. 2001). Furthermore, the stable environment and poor stable hygiene have also been implicated as a risk factor for respiratory disease. A poorly maintained and unclean stable environment may increase the magnitude of airborne challenges and substances which directly irritate airway mucosa (Newman-Taylor 1996). Lastly, strenuous physical activity may increase the risk of coughing. Racing, particularly on dry, dusty surfaces as seen more predominantly in the Standardbred industry, may aerosolise potential pro-inflammatory agents (Robinson and Slocombe 1986) and increase their rate of deposition in the airways. Racing and strenuous training may also act to influence the susceptibility of racehorses to respiratory disease, as strenuous exercise has a deleterious effect on the function of lung defence mechanisms (Wong, Thompson et al. 1990).

Causes of Lower Airway Inflammation

Infectious and non-infectious causes of LAI to which stabled horses may be exposed are considerable and include bacteria, viruses, moulds, parasites, allergens, irritants, noxious gases, and specific toxins such as endotoxin (see Figure 2). All these agents are potentially pathogenic due to their ability to initiate infection, induce allergy, behave as irritants or toxins or to overwhelm the efficiency of pulmonary defence mechanisms (Clarke 1992). Furthermore, a temporal relationship may exist between two or more of these agents (Bayly 2005), where it is possible that several agents may be required to act together for the development of LAI (Hodgson and Hodgson 2002).
Figure 2: Potential causes of lower airway inflammation in horses.

Non-Infectious Causes of Lower Airway Inflammation; Endotoxin
A variety of non-infectious inhaled environmental agents have been associated with induction of airway inflammation and include a range of organic and inorganic compounds such as viruses, moulds, mite debris and their faeces, plant material, β-glucans, inorganic dusts and bacterial endotoxins (Rylander 2001; Art, McGorum et al. 2002).

One important constituent of airborne particulate matter is endotoxin, which is one of the two most common of all naturally occurring molecules to which humans and animals are exposed (Rose 1973; Chetty and Schwab 1984). Endotoxin, or lipopolysaccharide (LPS), is a component of the wall of gram-negative bacteria (see Figure 3) and is liberated upon death of the bacteria. Endotoxin is ubiquitously present in airborne organic dusts and is prevalent in many occupational settings (Rylander and Jacobs 1994).

The vast majority of stable environments in which horses are housed are potentially contaminated with particulate matter with adherent bacteria and their cell-wall fragments, which may conceivably become aerosolised and subsequently inhaled. Furthermore, endotoxin is present in the oropharyngeal cavity (Jacobs 1997), nasal cavity (Michel 2000) and intestinal tract of human and animals, whereby aspiration of bronchial secretions may lead to bronchial contamination by endotoxin (Rosenthal and Tager 1975).

Under normal outdoor circumstances, low concentrations of endotoxin are inhaled and the lung has efficient defence mechanisms to counteract this airborne endotoxin. However, when high concentrations of dusts containing endotoxin is inhaled and deposited within the airways, inflammation develops (Malikides and Hodgson 2003). Evidence to support the role of endotoxin in the development of LAI in horses includes the observation that following inhalation of endotoxin, a dose-dependent neutrophilic inflammation is induced in horses that did not have any detectable pre-existing pulmonary disease (Pirie, Dixon et al. 2001). In addition, a significant linear relationship between average neutrophil percentage in lower airways and exposure to high endotoxin concentrations in breathing zone dust exists (Malikides 2003). Finally, endotoxin is known to engage the immune system in mammals, initiating physiological changes that appear to result in reduced performance and feed intake (Cargill, Skirrow et al. 1996).
The role of endotoxin in human occupational respiratory diseases is well documented (Douwes and Heederik 1997; Jacobs 1997). In accordance with these findings, animal studies have shown that inhaled grain dust containing endotoxin causes a profound neutrophilic response in the lower respiratory tract (Keller III, Lewis et al. 1987; Von Essen, Robbins et al. 1988) and that animals challenged with sufficient doses of respirable endotoxin will acutely recruit neutrophils to the lungs (interstitium, alveoli and airway) (Rylander and Beijer 1987; Gordon, Balmes et al. 1991).

Environmental & Stable Sources of Endotoxin
The environment in which racehorses are housed, and the management procedures to which they are exposed, pose a challenge to the requirements of optimal lung function during maximal performance (Bayly 2005). The air in stables differs greatly from the general environment, with the mean concentration of airborne particles in stables being more than ten times greater than that of outdoor air (Crichlow, Yoshida et al. 1980). The subsequent inhalation of various non-infectious agents has been proposed to cause airway inflammation, with all of these agents being commonly found in stable air and the training environment (Bayly 2005).

Within a horse stable, the primary sources of endotoxin and associated particulate matter are feed and bedding (Crichlow, Yoshida et al. 1980; Clarke and Madelin 1987; Clarke and Madelin 1987; Webster, Clarke et al. 1987; Woods, Robinson et al. 1993). As stable particle exposure and the stresses imposed on horses by training continue, these horses may subsequently progress to a point whereby they have severe airway inflammation, possible bacterial complications and clinical respiratory disease (Malikides and Hodgson 2003). In particular, young racehorses housed in confined stable areas where particulate matter is permitted to build up are potentially exposed to high levels of aerosolised non-infectious particles in a continuous and cumulative manner (Malikides and Hodgson 2003).

Clinical Signs Associated with Lower Airway Inflammation
A consistent observation in horses with LAI is the presence of a mucopurulent tracheal exudate containing neutrophils (Burrell 1985; Macnamara, Bauer et al. 1990; Sweeney, Humber et al. 1992; Moore, Krakowa et al. 1995). The increase in numbers and percentage of neutrophils within TA fluid (Chapman and Green 2000; Couetil, Rosenthal et al. 2001) is usually proportional to the severity of clinical signs and together these findings are often coupled with cough and decreased performance (Burrell 1985; Rush Moore, Krakowa et al. 1995). Other clinical signs of respiratory disease such as...
nasal discharge and pyrexia are infrequently associated with LAI (Burrell, Wood et al. 1996) and these horses usually have a normal attitude and appetite.

The effect of LAI on performance is controversial and is probably related to intensity of the inflammation. Although many horses with LAI do not have measurable alterations in lung function (Dixon, Railton et al. 1995), horses with more severe inflammation are likely to have uneven distribution of ventilation that would accentuate exercise-induced hypoxemia (Robinson 1997) and therefore result in decreased performance. In addition, although respiratory muscles consume only a small fraction of the total oxygen consumption of the body, as the respiratory system becomes less efficient as a result of disease, respiratory muscles may consume more oxygen and thus limit supply to the muscles of locomotion, resulting in a further limitation to the horse’s performance (Robinson; Persson 1983).

**Diagnosis of Lower Airway Inflammation**

Diagnosis of LAI is routinely conducted by use of endoscopic examination of the respiratory tract and collection of samples from the lower airways. Endoscopy is used to evaluate the colour of mucous membranes and the presence of abnormal secretions (e.g., mucus and/or blood) in the upper and lower airways (Derksen 1999). The collection of TA samples from horses using fiberoptic endoscopy has been described (Whitwell and Greet 1984; Darien, Brown et al. 1990; Martin, Beech et al. 1999), and this technique is now widely used for evaluating lower airway disease in horses, particularly performance horses, as minimal physical restraint and no chemical restraint is necessary. The presence of moderate to significant amounts of seromucoid or mucopurulent secretions in the tracheobronchial tree as visualised by endoscopy is consistent with pulmonary inflammation (Couetil 2002).

Lower respiratory tract samples are primarily collected for cytological and bacteriological evaluation. The two most common techniques used are TA and BAL which employ different techniques and sample different areas of the lungs. It is important to note that no significant correlation between BAL and TA cytology has been observed (Derksen, Brown et al. 1989; Malikides, Hughes et al. 2003), demonstrating that the cell population sampled by one technique are not representative of that obtained by the other. Therefore, results from either procedure cannot be extrapolated to the other. Tracheal aspirates were used in the current study and in a number of other large epidemiological studies in Australia and the United Kingdom, and therefore will be discussed solely in the remainder of this report.

**Treatment of Lower Airway Inflammation**

In recent times, there has been increased interest in mild and sub-clinical lower respiratory tract disease, particularly in Thoroughbred racing horses, as a cause of poor performance (Whitwell and Greet 1984; Burrell 1985; Burrell, Whitwell et al. 1994). However, treatment and management of young performance horses with LAI has remained largely anecdotal, with heavy reliance of recommended therapies of other, more chronic conditions found in older horses such as RAO. Nevertheless, conceptualisation of these important strategies needs to extend beyond these traditional forms of medication, with specific pharmaceutical goals for treatment for LAI in younger horses being reduction in the severity of airway inflammation, short term attenuation of cough, and maintenance treatments to prevent further exacerbations of disease (Hoffman 1997). In addition, successful treatment of LAI requires environmental management to minimize exposure to irritants (Rush 2002). Clearly, more defined strategies are needed to specifically obtain these goals for treatment of LAI in young performance animals.

**Pharmaceutical Intervention**

Lower airway diseases of horses are among the most common conditions encountered by equine veterinarians, however, few airway specific medications are available, with heavy reliance placed upon systemically or orally administered therapeutic agents (Derksen and Robinson 1998). Treatment of equine lower airway inflammation has traditionally included bronchodilatory and anti-inflammatory
drugs (Derksen 1991), with corticosteroids being the most effective of the anti-inflammatory agents in clinical use due to its widespread effects on the inflammatory response (May 1992). Corticosteroids are routinely administered by veterinarians, both systemically and locally, for their anti-inflammatory properties in the horse. Although the efficacy of systemic treatments is proven (Klein and Deegan 1985; Broadstone, Scott et al. 1988; Pearson and Riebold 1989; Lapointe, Lavoie et al. 1993), orally administered corticosteroids such as prednisolone require systemic absorption and circulation to be effective in the treatment of respiratory diseases. However, systemically absorbed corticosteroids can produce a number of undesirable effects such as muscle wasting, hyperglycaemia, polydipsia, polyuria, laminitis and immunosuppression (Eyre, Elmes et al. 1979; Macharg, Bottoms et al. 1985; Cohen and Carter 1992). In addition, administration of corticosteroids to horses in high doses or for extended periods can lead to more severe adverse systemic effects, including adrenal insufficiency, iatrogenic Cushing’s syndrome, immune suppression and laminitis (Harkins, Carney et al. 1993; Ferguson and Hoenig 1995).

To address the adverse reactions associated with systemic corticosteroid therapy, there has been increased interest in the aerosol mode of delivery which has the advantage of specifically targeting pulmonary drug delivery and decreasing systemic effects, while simultaneously increasing biological availability of the administered drug through local deposition in the lungs.

**Aerosol Therapy**

Inhalation treatment of lower respiratory tract disorders in humans date back at least 4,000 years to India, when *Atropa belladonna* leaves were smoked for relief of cough (Grossman 1994). In the present day, numerous parallels can be drawn between small airway diseases in horses and humans and therefore lessons in therapeutic interventions can be drawn. Although aerosol therapy is the gold standard for the control of asthma in humans, its use in veterinary medicine is still in the developmental stages, although there are several *sine qua non* conditions for its accurate administration in large animal medicine (Lekeux 2000).

Devices which deliver drugs from metered dose inhalers (MDI’s) emerged in human medicine 37 years ago, though interest in their use for delivery of medications in horses has surged only in the last decade (Hoffman, Viel et al. 1993; Tesarowski, Viel et al. 1994; Hoffman 1995). Interestingly, the characteristics of the equine airways and patterns of breathing are well suited to inhalation therapy, as the large tidal volume, high flow rate and obligate nasal breathing of the horse encourage aerosol deposition deep within the lung. Significantly aiding an increase in the use of aerosol therapy in the horse has been the introduction of several appliances including the equine Aeromask™ and Equinehaler™ which have been specifically designed for MDI administration (Duvivier, Votion et al. 1997). As a direct result of these technical advances, effective delivery of drugs to the equine respiratory system by aerosol inhalation is now a viable alternative to the systemic administration of the same agents.

Experimental data has established that aerosolised drug therapy is an efficient means of treatment for young horses with LAI (Rush 2002; Bayly 2005; Bayly 2005). Inhalation therapy confers a number of advantages over systemic therapy including the observation that it enables higher concentrations of the drug to be deposited specifically in the airways, while at the same time, requiring a lower total dose than if the medication is delivered parenterally (Lekeux and Duvivier 1996). Thus, inhalation therapy improves drug safety and efficacy by reducing the total therapeutic dose, minimising drug exposure to other body systems and allowing the direct delivery of the drug to the lower respiratory tract (Rush 2002).

**Aerosol Therapy Delivery Devices**

Early devices for equine inhalation therapy and the delivery of aerosolised drugs to the lower respiratory tract were expensive, unwieldy and only marginally efficacious. This is in stark contrast to the systems available today, allowing inhalation therapy to become an increasingly popular treatment of lower respiratory tract disease in horses (Rush 2002).
Therapeutic aerosols are commonly produced by atomization of liquids within a jet or pneumatic nebuliser, by vibration of a standard liquid pool such as in ultrasonic nebulisation (UN). Alternatively, pre-formed aerosols may be administered via metered dose inhalers (MDI) and dry powder inhalers (DPI) (Lekeux and Duvivier 1996). However, depending on the administration device, the relative proportions of delivered drug to the small airways may vary significantly between ultrasonic nebulisers, jet nebulisers, metered dose inhalers and dry powder inhalers, which have all been shown to have acceptable, albeit variable, deposition characteristics in the equine lung (Geor and Johnson 1993; Duvivier, Chiap et al. 1997; Votion, Ghafir et al. 1997; Davis 1998).

**Metered Dose Inhalers (MDI)**
Metred Dose Inhalers are hand-held devices which contain active drugs released in conjunction with a high pressure excipient, and are classically referred to as ‘puffers’, as is the case with human asthma medication preparations. Advantages of an MDI include rapid administration, consistent dose delivery, minimal risk of pulmonary contamination with environmental organisms, low maintenance of equipment, wide availability, access for ‘per need’ administration and no requirement for electricity (Rush 2002; Bayly 2005). Moreover, a radioaerosol study conducted by Viel and colleagues (Viel and Tesarowski 1994) demonstrated superior (five fold) deposition of MDI particles in the lung when compared with standard nebulisation.

**Aerosol Coupling Devices**
The two most common coupling devices enabling the use of MDI’s in horses are the Aeromask™ and Equinehaler™. Both of these devices are fitted with a one way inspiratory valve attached to a low dead space face mask designed for safe and effective aerosol drug delivery in the horse (Duvivier, Votion et al. 1997). The MDI is actuated into the spacer from which the drug is inhaled, essentially providing breath actuated delivery of MDI aerosols and minimal condensation of the drug (Hoffman 1997). When employed correctly, this system provides a potent delivery of drugs to horses with lower airway inflammation such as RAO or LAI (Hoffman, Viel et al. 1993; Tesarowski, Viel et al. 1994). Furthermore, this devices increases lung deposition of aerosolised drug as they hold a rich cloud of small particles while preventing condensation from exhaled vapour and simultaneously minimising problems associated with malposition.

![Figure 4: Inhalation therapy using the Equinehaler™ (Equine Healthcare, APS, Hillerod, Denmark)](image)
The Equinehaler™ (see figures 4 and 5) is an inhalation ‘spacer’ device with a nasal mask designed specifically for accurate administration of pharmaceuticals to horses with inflammatory respiratory diseases such as RAO or LAI. The apparatus is adapted to fit entirely over the left nostril and provides a convenient mechanism for administration of all available types of MDI’s to horses ensuring the delivering a suitable particle size (<5µm) for direct distribution to the small airways. A study conducted by (Funch Nielsen, Roberts et al. 2001) investigated the efficacy of the Equinehaler™ for the delivery of fluticasone propionate (Flixotide®: 250µg/actuation) from an MDI into the equine lung, while also determining the pulmonary distribution. In vitro studies demonstrate that the mean particle size distribution (PSD) of fluticasone and radiolabelled 99mTechnetium are similar, indicating the deposition of the radiolabel within the lungs is likely to reflect that of fluticasone. Mean lung deposition for all animals was shown to be 8.2 ± 5.2% of the administered dose.

Unlike the Aeromask™, the Equinehaler™ is able to accommodate any sized horse without need for creating an airtight seal over the muzzle (Bayly 2005). However, caution must be taken to ensure the administrator aligns the MDI with the spacer device and the spacer apparatus with the nasal passages of the horse during actuation, as malpositioning may result in poor pulmonary drug delivery (Bayly 2005). In addition, movement of the head or alteration of breathing pattern in response to actuation of the MDI can detract from pulmonary drug delivery (Bayly 2005).

**Figure 5:** The Equinehaler™ Aerosol Delivery Device

**Pharmaceutical Agents Used for Treatment of Lower Airway Inflammation**

**Corticosteroids**
Corticosteroids are one of the most effective anti-inflammatory agents in clinical use. They affect all aspects of the inflammatory response (May 1992) and subsequently are routinely administered by veterinarians systemically and locally for their anti-inflammatory properties in the horse. However, administration of corticosteroids systemically to horses in high doses or for extended periods can lead to severe adverse systemic effects, including adrenal insufficiency, iatrogenic Cushing’s syndrome, immune suppression and laminitis (Harkins, Carney et al. 1993; Ferguson and Hoenig 1995).

In human medicine, inhaled corticosteroids are highly effective in the control of asthma, as they exert a topical effect in the lungs but become inactivated when absorbed from the gut (Lekeux and Duvivier 1996). In horses with lower airway disease, improvement of clinical signs following systemic corticosteroid therapy has long been observed and therefore aerosol administration of these agents is of particular interest but needs further investigation (Lekeux and Duvivier 1996).

In collaboration with environmental changes, corticosteroids may decrease the time period for clinical remission to ensue, but, long term corticosteroid use in the absence of environmental control will result in incomplete remission of clinical signs (Dixon 1992). In addition, although inhaled corticosteroid therapy is associated with a low risk of systemic effect compared to oral or parenteral
administration, systemic absorption of corticosteroid still occurs (Goldberg, Algur et al. 1996; Yiallouros, Milner et al. 1997; Mollmann, Wagner et al. 2001).

Fluticasone propionate is an androstane, synthetic trifluorinated corticosteroid which has the most potent anti-inflammatory activity of the commercially available aerosolised corticosteroid preparations (Rush 2001). Corticosteroid therapy, and in particular the corticosteroid fluticasone propionate, is well suited to inhalation therapy in the horse because of the large number of glucocorticoid receptors on both bronchial epithelial and vascular endothelial cells in this species (Lavoie 1998). In addition, inhaled fluticasone propionate has been shown to have a positive therapeutic effect in horses by resolving airway inflammation (Giguere, Viel et al. 2002).

Bronchodilator Therapy
Aerosolised bronchodilators provide immediate relief of airway obstruction, provide protection against irritant-induced bronchoconstriction and are an important component of treatment of non-infectious respiratory disease (Bayly 2005), but, they do not influence the inflammatory changes in the smaller airways. In addition, similar to corticosteroids, they do not address the underlying cause of the airway inflammation and are therefore only palliative. Administration of bronchodilators prior to exercise may aid in preventing exercise-induced bronchoconstriction (Rush 2002), and administration of bronchodilators immediately prior to administration of topically acting corticosteroid preparations is thought to improve pulmonary drug distribution and prevent irritant cough and bronchoconstriction (Rush, Hoskinson et al. 1999; Bayly 2005).

Detection of Common Veterinary Pharmaceuticals
The development of more sensitive testing methods, necessary to detect new and sophisticated performance enhancing drugs, has resulted in the potential to detect miniscule quantities of therapeutic medications. As the methodology of analytical drug testing has improved, smaller quantities of drugs and medications are able to be detected in urine samples collected after races and other athletic events (Kollias-Baker 2002) and therefore it is accepted that advice concerning acceptable veterinary drug treatment practices also should be available.

It is also expected that this advice would be supported by data that enables veterinarians to treat horses, whilst also complying with the rules of racing. However, there is minimal reliable drug excretion data in the scientific literature that is relevant to drugs used for treating lower airway inflammation and therefore can be of help to the racing industry as a guide to legitimately treat horses with this disorder.

Conclusions
Although LAI is highly prevalent in young performance horses, and is likely to have detrimental effects on performance, clear strategies and guidelines for treatment and prevention have not been well established. Therefore, it was the aim of this study to identify improved management techniques to decrease exposure to the agents commonly involved with induction of LAI, such as dust associated endotoxin. A second aim was to determine recommendations regarding use of aerosolised medications for treatment of LAI in young performance horses. This was achieved by performing tightly controlled clinical trials conducted ‘in field’ i.e., where horses were subjected to standard management practices. A final goal of the current research was to provide reliable and up to date drug excretion data on inhaled aerosolised agents in order to provide the racing industry with information that would potentially allow the legitimate veterinary use of drugs for treatment of LAI.
STUDY 1: Total and Respirable Particle Endotoxin Concentrations Generated by Specific Feed and Bedding Materials

Introduction
Young racehorses housed in confined stable areas are potentially exposed to high concentrations of aerosolised non-infectious particulate matter containing endotoxin. Endotoxin is a heat-stable lipopolysaccharide complex present in the cell wall of gram-negative bacteria and is liberated when these bacteria die or multiply (Rietschel and Brade 1992). Airborne endotoxin is ubiquitous in many agricultural environments including dairy farms, buildings housing swine, poultry sheds, animal farm bedding materials and stables (Olenchock, May et al. 1990; Liesivuori, Kotimaa et al. 1994; McGorum, Ellison et al. 1998; Malikides, Christley et al. 2003). Inhalation of this molecule results in pro-inflammatory responses in animals and man. In particular, horses housed in stables are susceptible to the effects of inhaled endotoxin such as a neutrophilic infiltratation and tracheal mucus accumulation, which are both risk factors for poor racing performance (Holcombe, Robinson et al. 2004). Furthermore, as stable particle exposure continues, in conjunction with the other stressors imposed by training, these horses may subsequently progress to a point whereby they have significant airway inflammation, possible bacterial complications and clinical respiratory disease.

Within a horse stable, the primary sources of endotoxin and associated particulate matter are feed and bedding (Crichlow, Yoshida et al. 1980; Clarke and Madelin 1987; Clarke and Madelin 1987; Webster, Clarke et al. 1987; Woods, Robinson et al. 1993). However, preliminary studies have demonstrated that endotoxin concentrations within mixtures of feeds and certain bedding types are highly variable. Therefore, information on the contribution of individual feeds and bedding types to the exposure of horses to particulate matter and endotoxin within their breathing zone would be highly relevant, as minimal increases in endotoxin exposure may have substantial adverse effects on respiratory tract health and performance.

Preliminary Studies
A number of studies have investigated respirable and total particulate matter in the breathing zones of horses in addition to concentrations of endotoxin in these samples (McGorum, Ellison et al. 1998; Malikides, Pike et al. 2000; Malikides, Christley et al. 2003; Pirie, Collie et al. 2003). These studies have validated the equipment for particle collection (e.g., programmable pumps and particle filters), assessed acclimation and tolerance of horses to collection of particle samples, explored the methodology of a commercial kit for measurement of particle endotoxin (Endospecy (ES-50M) kit; Limulus Amoebocyte Lysate [LAL], Sapphire Biosciences, Crows Nest Australia) and determined the concentrations of endotoxin in the breathing zones of horses housed in looseboxes. Therefore, the methodologies applied in these studies were used as a foundation for the materials and methods in the current study.

Objectives
The purpose of this study was to determine the potential concentrations of airborne particles and particle endotoxin generated by specific feeds and bedding materials within the breathing zone of the stabled horse. In addition, the study aimed to provide a critical assessment of endotoxin concentrations in relation to threshold limiting values (TLVs) reported within the published literature. Thus, we aimed to provide recommendations regarding the selection of feed and bedding materials, coupled with feasible management strategies, which may aid in reducing airborne endotoxin concentrations within the stable environment.
Methodology
Exposures to breathing zone particles (dust) and particle endotoxin generated by four feeds (oats, wheaten chaff, lucerne hay, pellets) and four bedding materials (high quality straw, rice hulls, sawdust, coarse shavings) were each assessed separately on six occasions using a crossover Latin Square design. These feeds and beddings were chosen as they were the most widely used throughout Sydney metropolitan racing stables. The cross-over design and subsequent analysis assumes no carry-over effects or equivalently, that they are removed by any ‘wash out’ between treatment periods (Ratkowsky, Evans et al. 1993). One horse was used for collection of all samples in these experiments to address inter-horse variation as a confounding factor.

Total and respirable particle samples were collected simultaneously from the breathing zone of a six year old SB gelding via personal air sampling (PAS) devices attached to alternate sides of the head collar approximately 15 centimetres (cm) caudal to the nares (see Figure 6). The device consisted of a programmable vacuum pump (224-PCXR8, Airmet Scientific Pty Ltd, Nunawading, Australia), polycarbonate filter (25mm diameter and 0.8µm pore size) for adherence of particulate matter (Millipore, Sydney Australia), a filter holder and coiled latex rubber tubing attaching the pump to the filter holder with protective cowl (Anon 1980). For total particle collection, an open-faced filter holder (Institute of Occupational Medicine [IOM] attachment, Worksafe®, Australia) was used. For respirable particle collection, a cyclonic impactor (Higgins Cyclonic Impactors, Casella, London) was used.

The stable environment was modified by enclosing all walls with heavy-duty poly vinyl chloride (PVC) sheeting in order to prevent external confounding factors, such as wind speed, changes in ventilation and entry of external airborne particles, to create a static sampling environment. This modification permitted determination of the specific individual contributions of each feed and bedding material to particulate matter and particle endotoxin concentrations within the stable.

The same horse was used for the duration of the experiment, following an adequate (2 ±1.5 days) acclimation to the stable, PAS devices and feeding regimen. The experimental stable was thoroughly cleaned and rested for a minimum of three hours between sampling times prior to the randomised introduction of new bedding or feed.

Figure 6:  Personal Air Sampling (PAS) Equipment used for Collection of Total and Respirable Particles in Various Feeds and Beddings

A uniform litter depth of 30cm across the stable floor area (3.6 metres (m) x 3.6m) was used for each bedding type to ensure adequate coverage of the stable floor. The duration of sampling for each of the four bedding materials was one hour exposure time, with sampling time for each of the four feeds varying according to the number of minutes required for complete consumption (oats 21±5.85, lucerne
hay 36±8.28, wheaten chaff 88±48.37, pellets 15±5.79). The horse was provided with one kilogram of each of the individual feeds.

Following sample collection, total and respirable particle samples on filters were weighed and stored in micro-petri dishes in a darkened refrigerator before commencing batch analyses. Particles were extracted from collection filters and assayed for soluble endotoxin concentrations using a commercial endotoxin-specific assay kit involving the Limulus Amoebocyte Lysate (LAL) test; (Endospecy, Seikagaku Co., Tokyo, Japan) as it had been previously employed for equine particle filter analysis (McGorum, Ellison et al. 1998; Malikides 2003). Detailed analytical methods have been described for this procedure (Olenchock, Murphy et al. 1992). Following completion of analysis, the absorbance of the samples was read spectrophotometrically at 405 nanometres (nm) for a resultant colour change, indicating endotoxin concentration within individual samples.

In order to calculate the average concentration of respirable or total particles in milligrams per cubic metre of air (mg/m³), the following formula was used;

\[
Concentration = \frac{m}{V}
\]

Where:
- \( m \) = mass of particles on filter, in mg
- \( V \) = volume of air passed through the filter, in m³ and is equal to the sampling time (minutes) × the average flow rate of cyclone (L/minute).

As a consequence of dilution difficulties with the endotoxin (LAL) assay, only crude filter weight data (total and respirable dust) for rice hulls were included in the statistical analyses.

Statistical Methods

One way unbalanced analysis of variance (ANOVA) was performed using the Genstat® 8th Edition statistical software package. Analysis was performed on total and respirable particle concentration data and log-transformed endotoxin concentration data obtained for each of the feeds and each of the beddings. Significance was ascribed at the \( p<0.05 \) level.

Results

Mean total and respirable particle and total and respirable particle endotoxin concentrations to which horses may potentially be exposed when standing on different bedding materials and eating different feedstuffs are presented in Tables 1 and 2 respectively.

**Table 1: Mean (±SE) Total and Respirable Particle Exposures (mg/m³) and Particle Endotoxin Exposures (ng/m³) Generated from Four Different Stable Bedding Materials (n=6 for dust; n=2-5 for endotoxin).**

<table>
<thead>
<tr>
<th>Bedding</th>
<th>Total Dust (mg/m³)</th>
<th>Total Endotoxin (ng/m³)</th>
<th>Respirable Dust (mg/m³)</th>
<th>Respirable Endotoxin (ng/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straw</td>
<td>19.4±3.2^</td>
<td>92,263±43,082^</td>
<td>8.8±1.4^</td>
<td>2,918±834^</td>
</tr>
<tr>
<td>Shavings</td>
<td>8.2±2.7</td>
<td>1,227±284</td>
<td>1.5±0.4</td>
<td>728±394</td>
</tr>
<tr>
<td>Sawdust</td>
<td>3.5±0.7</td>
<td>1,397±99</td>
<td>2.2±0.4</td>
<td>229±142</td>
</tr>
<tr>
<td>Rice hulls</td>
<td>132.5±18.8*</td>
<td>620,524±169,616#</td>
<td>39.3±5.8*</td>
<td>122,737±61,099#</td>
</tr>
</tbody>
</table>

* Significantly higher than other bedding types (One-way ANOVA; \( P<0.05 \))
^ Significantly higher than sawdust and shavings (\( P<0.05 \))
# Not included in analysis.
Table 2: Mean (±SE) Total and Respirable Particle Exposures (mg/m³) and Particle Endotoxin Exposures (ng/m³) Generated from Four Different Feeds (n=6 for dust; n=2-5 for endotoxin).

<table>
<thead>
<tr>
<th>Feed</th>
<th>Total Dust (mg/m³)</th>
<th>Total Endotoxin (ng/m³)</th>
<th>Respirable Dust (mg/m³)</th>
<th>Respirable Endotoxin (ng/m³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oats</td>
<td>6.6±3.1</td>
<td>2,405±1,154</td>
<td>4.4±1.1</td>
<td>154±101</td>
</tr>
<tr>
<td>Lucerne Hay</td>
<td>12.1±3.1</td>
<td>7,291±3,187</td>
<td>4.3±1.1</td>
<td>374±230</td>
</tr>
<tr>
<td>Pellets</td>
<td>9.6±4.1</td>
<td>1,762±679</td>
<td>7.5±2.9</td>
<td>405±163</td>
</tr>
<tr>
<td>Wheaten Chaff</td>
<td>4.2±0.7</td>
<td>2,128±494</td>
<td>2.8±1.2</td>
<td>516±240</td>
</tr>
</tbody>
</table>

**Bedding Experiment**
The average exposure to breathing zone total and respirable dust particles generated from rice hulls was significantly higher than all other bedding types (p<0.05). In addition, the average exposure to breathing zone total and respirable particles generated from straw was significantly higher than either shavings or sawdust (p<0.05). Total and respirable endotoxin concentrations generated by straw was significantly higher than those produced by shavings and sawdust (p<0.05), but total and respirable endotoxin concentrations produced by rice hulls could not be included in the statistical analyses due to problems associated with dilution of these samples. However, the data would suggest that these concentrations were significantly greater than those produced by all other bedding types. There was no significant difference between shavings and sawdust for either total or respirable dust particles or total or respirable endotoxin concentrations.

**Feed Experiment**
There were no statistically significant differences between concentrations of total and respirable dust particles, and total and respirable particle endotoxin concentrations in the breathing zone of exposed horses generated by the four feed types. Each feed type analysed in this experiment appeared to produce a similar concentration of airborne endotoxin over each individual exposure time. However, although no significant difference was found between feeds, total endotoxin concentrations in all feeds exceeded total concentrations generated by both shavings and sawdust. In contrast, beddings exceeded feeds for respirable endotoxin concentrations for all feed types except oats. In addition, due to the relatively short time frame the horse is exposed to feeds as opposed to the continuous and cumulative exposure provided by bedding materials, feed type was not found to be a significant factor (p = 0.294) in contributing to stable aerosol endotoxin concentrations, although consideration must still be given to the selection of low endotoxin generating types of feed.

No linear correlation was found between total and respirable particulate matter concentrations and their respective counterpart total and respirable endotoxin concentrations. Therefore it was concluded that gross particulate matter concentrations (or visual estimates of these) cannot be used as a surrogate measurement of anticipated endotoxin concentrations in future experiments.

**Discussion and Conclusions**
The endotoxin concentrations in total and respirable particles detected in the current study were significantly higher when horses were bedded on straw and rice hulls, than when bedded on sawdust and shavings, and is in agreement with findings from previous studies (McGorum, Ellison et al. 1998; Malikides, Pike et al. 2000). Furthermore, stalls containing straw bedding evaluated in other studies have been found to have significantly higher concentrations of total and respirable airborne endotoxin contamination as compared to stalls containing phone book paper and sawdust (Vandenput, Istasse et al. 1997; Tanner, Swinker et al. 1998; Ward, Wohlt et al. 2001). However, as with straw, if these materials are used in deep litter systems or in warm and poorly ventilated stables, they also may become a significant source of endotoxin (Clarke 1987; Art, McGorum et al. 2002).
Compared with bedding types, feed types investigated in the current study did not produce significantly different concentrations of respirable endotoxin and were found not to be a significant factor in contributing to stable aerosol endotoxin concentration. These findings are in conflict with earlier studies where traditional feedstuffs, such as hay, increased the concentration of endotoxin in the horse’s breathing zone by up to six-fold greater than low dust alternatives such as pellets (Art, McGregor et al. 2002). Although no significant differences were found between the four feed types assessed in the current study, all four feed types were found to generate endotoxin concentrations above the threshold limiting value (TLV) of respirable endotoxin known to be associated with the induction of airway inflammation in humans (> 4.5 to 10 ng/m$^3$ to induce detectable airway inflammation and > 100 to 200 ng/m$^3$ to induce clinical respiratory signs and disease) (Rylander 1997). In the case of horses, reports vary as to the TLV of endotoxin required to produce lower airway inflammation. In one study inhalation of 20-200µg of aerosolised endotoxin experimentally induced increased numbers of neutrophils in the lower airways (Pirie, Dixon et al. 2001). As 20 to 200 µg is approximately equivalent to 430 to 4300 ng/m$^3$, in the current study wheaten chaff was the only feed which produced respirable endotoxin concentration exceeding the lower value (516±240 ng/m$^3$), though lucerne hay and pellets produced values which were close (374±230 and 405±163 ng/m$^3$ respectively). In another study, respirable endotoxin concentrations > 4.2 ng/m$^3$ in a stable environment were associated with significant lower airway neutrophilia (>20%) in dose-dependent manner (Malikides 2003), and the concentrations of all feeds used in the current study are significantly higher than this concentration. Finally stabled horses spend 39% of their time eating forage and concentrates (Thompson 1995), and therefore, although feed type was not being found to be a significant contributor to stable endotoxin burden, the contribution of feeds to inhaled particulate matter and respirable endotoxin within the breathing zone cannot be discounted. In addition, concentrations of total endotoxin produced by all bedding types and respirable endotoxin produced by straw, shavings and rice hulls exceeded the TLV for induction of inflammation in horses, and in the case of rice hulls this value was greatly exceeded.

This research provides the first data to quantify potential endotoxin exposures generated from individual feeds and beddings in a controlled stable environment, whereby the experimental subject was not exposed to feed and bedding sources concomitantly. Results from this study indicate that horses are potentially exposed to very high concentrations of breathing zone particles and particle endotoxin generated by specific bedding types. Furthermore, results may be interpreted to suggest that differences in endotoxin concentrations between beddings are highly variable. In contrast, less variation was observed between different feed preparations, and these appear to contribute less to endotoxin concentrations, particularly inspired endotoxin, in the horse’s stable environment. Therefore, this study has established that appropriate choice of bedding type can significantly decrease endotoxin concentrations in stables, and therefore may be an effective method of reducing the incidence and prevalence of airway inflammation in stabled horses.
STUDY 2: Inhalation Therapy Pilot Study

Comparative Efficacy of Inhaled Aerosol Therapies (ipratropium bromide, fluticasone propionate and combination therapy) for the Reduction of Pulmonary Neutrophilia Associated with Lower Airway Inflammation in Horses

Introduction

Lower airway inflammation is a highly prevalent condition and is potentially detrimental to the performance of young race and performance horses. Long term amelioration of the syndrome requires minimising exposure of the horse to pro-inflammatory inhaled agents. However, in the absence of such environmental control measures, or in association with these, short-term benefits may be gained via use of pharmaceutical agents.

Current research has established airway inflammation occurs in 22-50% of Thoroughbred and Standardbred racehorses and is considered a common cause of impaired performance and interruption to training (Burrell 1985; Macnamara, Bauer et al. 1990; Sweeney, Humber et al. 1992; Malikides 2003). Mainstay therapies for inflammation of the lower respiratory tract are systemically administered bronchodilators and anti-inflammatory drugs, usually corticosteroids. However, the recent availability of tailored systems for aerosol drug administration in horses has improved the safety and efficacy of this treatment. These devices optimise the topical delivery and deposition of high concentrations of the inhaled drug at target receptor sites within the lungs whilst simultaneously minimising the undesirable side effects commonly encountered when these drugs, particularly corticosteroids, are administered parenterally. Additionally, the characteristics of the equine airways and patterns of breathing are well suited to inhalation therapy, as the large tidal volume, high flow rate and obligate nasal breathing of the horse encourage aerosol deposition deep within the lung.

To date, minimal research has been conducted comparing the individual efficacy of aerosol drug preparations in young performance horses with lower airway inflammation. This study therefore aimed to compare the treatment regimen efficacy of an inhaled bronchodilator, corticosteroid and combination of the two in reducing pulmonary neutrophilia associated with lower airway inflammation in horses. In addition, a control group was also included so as to ascertain the natural progression of inflammation during the trial period.

Objectives

To determine if inhalation therapy using either a bronchodilator, corticosteroid or combination of these drugs, provides resolution of airway inflammation as determined by pulmonary neutrophilia. Furthermore, this experiment was used as a pilot study for later investigation of these therapies in a large, randomised clinical trial.

Methodology

The pilot study was conducted at the University of Sydney horse unit, Camden. Sixteen horses of mean age (9.5 ± 4.5) and varied breed (Thoroughbred and Standardbred) with cytological evidence of lower airway inflammation were enrolled in the 14 day aerosol therapy study. Horses exhibiting >20% neutrophils and less than 5% eosinophils along with increased tracheal mucus were allocated to the trial. All horses were maintained at pasture for the duration of the study period.

Enrolled horses were randomly allocated to one of four treatments (n=4); Flixotide® (fluticasone propionate [steroid]) 1,000µg; Atrovent® (ipratropium bromide [bronchodilator]) 250µg; Combination (Atrovent® + Flixotide®) 250µg Atrovent® 30 minutes prior to 1,000µg Flixotide®; Control [placebo]. Treatments were administered twice daily (q12h) via a metred dose inhaler (MDI)
with an Equinehaler® used as a coupling device. A total of eight horses were used in the trial, and each horse was treated with two, out of a possible four treatments whereby there was a 27 day wash out between treatments to ensure no carry over effects of the previously administered treatment.

The lower respiratory tract health of enrolled horses was assessed on Day 0 via endoscopy and tracheal aspirate (TA) sample collection. Tracheal aspirates were collected using a standard endoscopic technique and with a guarded catheter (Christley 1999; Malikides 2003; Robinson, Berney et al. 2003) to facilitate cytological and bacteriological evaluation of the collected sample. Further collection of TA samples via an endoscopically-guided guarded catheter occurred on Day 4, Day 7, Day 11 and Day 14 (cessation of treatment). Ten ml of sterile saline was inoculated into the trachea of each horse, and following aspiration of greater than 6mL of instilled fluid, suction was ceased, the catheter was retracted into the biopsy channel and endoscope slowly removed from the respiratory tract in a downward motion. The syringe containing the TA was placed on ice and transported to the laboratory within one hour of collection.

Approximately two mL of the TA sample was transferred in a sterile manner into a sterile two mL vial for bacteriological analysis. The remainder of the sample (usually > 4mL) was placed into a labelled non-sterile container for cytological analysis. The results of differential TA cytology and relative reduction in neutrophil cell counts were incrementally compared between Day 0, Day 4, Day 7, Day 10 and Day 14 of treatment in order to determine comparative treatment efficacy for the resolution of pulmonary neutrophilia.

**Statistical Methods**

Data was recorded in an Excel spreadsheet and analysed using GenStat, Version 8. Differences between each of the four treatments for neutrophil percentages were analysed. Due to the unbalanced nature of the data, Analyses of Variance (ANOVA) was performed using the Unbalanced Treatment Structure (UTS) option, where ‘horse’ was considered to be a block effect (or replicate). This analysis was compared to a Completely Randomised Design (CRD), in which no block effect (or ‘horse’ effect) was included. Statistical significance was reported at the 5% (0.05) level.

**Results**

There was a significant difference between treatment groups in their efficacy for decreasing pulmonary neutrophilia over the course of therapy (p=0.044). From Day 0 to Day 7 there was a marked increase in proportions of neutrophils in TAs collected from control horses (mean = +34.5%) whilst there was a decrease in proportions of neutrophils for all three treatments (-0.5%, -1.2% and −9.0% for Atrovent, Flixotide and the Combination respectively) (see Figure 7). From Day 7 to Day 14, there was a further decrease in proportions of neutrophils for the three treatment groups (-8.8%, -20.8% and −9.5% respectively). However, during this time period the control group also experienced a decrease in proportions of neutrophils (-20.2%) (see Figure 7). Although the control groups proportions of neutrophils decreased during this second time frame, the proportions of neutrophils remained above the cut off mark used to determine the presence of lower airway inflammation (ie >20% neutrophils in TA secretions) in these horses.

If results from Day 0 to Day 14 of the study are compared, horses receiving the placebo control demonstrated an overall increase percentage of neutrophils (+14.2%) over the complete treatment period, compared to a decrease in percentage of neutrophils in those horses treated with the Combination (-18.5%), Flixotide® (-22.0%) and Atrovent alone (-7.9%) (see Figure 7). This represented a significant difference in the percentage of neutrophils between the placebo control and the Combination therapy and Flixotide alone (p=0.012). However, there was no statistically significant difference between the placebo control and the Atrovent® groups (p=0.82) indicating the use of a bronchodilator alone for the treatment of airway inflammation is ineffectual.
When assessing incremental changes in neutrophil percentages during the course of the study, there is a highly significant difference between treatments between Day 0 and Day 7 in reduction of percent neutrophils ($p=0.003$). However, no significant difference was found between treatments ($p=0.583$) between Day 7 and 14 in reduction of percent neutrophils. This finding is likely a result of the decreasing proportions of neutrophils in the control group during this time, and does not necessarily reflect the ongoing efficacy of treatments (Combination, Flixtotide and Atovent) over the entire time of the study.

![Changes in Neutrophil Percentages](image)

**Figure 7:** Graphical Summary of the Changes in Neutrophil Percentage over Time (Day 0, Day 7, Day 14) for the four Treatment Groups

**Discussion and Conclusions**

Results from the pilot study suggested that the assessed therapies were not equally effective for the treatment of pulmonary neutrophilia in horses. Overall, a significant difference was found between the four treatments ($p=0.044$) over the course of therapy and reflected the variation between the three treatment groups and control horses. Horses receiving ipratropium bromide (Atrovent®), fluticasone propionate (Flixtotide®) and a combination of these drugs all showed an overall reduction in percent neutrophils of -9.2%, -22.0%, and -18.5% respectively. However, although there was not a statistical difference between these three treatments, there was a trend towards significance for results from horses receiving ipratropium bromide (Atrovent®) therapy when they were compared to horses receiving fluticasone propionate (Flixtotide®) or a combination of fluticasone and ipratropium. This was further reflected in the observation that there was no statistically significant difference between the control group and the group of horses receiving Atrovent® therapy ($p=0.82$) indicating that stand alone bronchodilator therapy is not efficacious for treatment of lower airway neutrophilia in horses on pasture.

When results were evaluated over time, there was a highly significant difference between treatment groups when samples from Day 0 and Day 7 were compared for a reduction in the proportions of neutrophils ($p = 0.003$). In contrast, no significant difference was found between treatments when samples from Day 7 and Day 14 were compared ($p = 0.583$). This result most likely reflects the decrease in proportions of neutrophils observed in samples obtained from the control group, which masked the effect of the ongoing decrease in proportions of neutrophils in the groups receiving fluticasone or a combination of fluticasone and ipratropium.
In conclusion, on the basis of this pilot study, the use of the corticosteroid fluticasone propionate and a combination of fluticasone propionate with the bronchodilator ipratropium bromide appear to be equally efficacious for treatment of pulmonary neutrophilia in horses on pasture. In contrast, the use of bronchodilator (ipratropium) therapy alone does not appear to be effective for reducing airway inflammation. Although these results suggest the efficacy of the combination therapy is likely to be due to the effect of fluticasone alone, other inhalational studies have shown that when a bronchodilator is administered shortly before a corticosteroid, there is better penetration of the corticosteroid deep within the lower airways due to a bronchodilatory effect. This has resulted in improved treatment outcomes. Therefore, the drugs recommended for evaluation of clinical efficacy in a larger, randomised trial were fluticasone propionate (Flixotide®) and a combination of fluticasone propionate (Flixotide®) and ipratropium bromide (Atrovent®)

Recommendations for the length of time of treatment were less clear. Although there was a significant effect of treatment from Days 0 to 7, this was not continued from Days 7 to 14. However, as discussed previously, this is likely a reflection of the decrease in proportions of neutrophils observed in the control (placebo) group and does not necessarily indicate a decrease in an ongoing effect of the drugs under consideration. Therefore, it was suggested that a second week of therapy could provide additional improvement in decreasing pulmonary inflammation and should be recommended for the Randomised Clinical Trial.
STUDY 3: Inhaled Medication Drug Detection Studies

Section 3A: Detection of the Inhaled Ipratropium Bromide (Atrovent®) in Equine Urine using Liquid Chromatography Mass Spectrometry (LC/MS).

Introduction
Ipratropium bromide (Atrovent®) is an anticholinergic drug which acts by blocking acetyl choline receptors on smooth muscle, including muscle fibres present in the trachea and large airways, resulting in muscle relaxation and bronchodilation (Derksen and Robinson 1998). When this drug is administered via inhalation therapy, high concentrations of the drug are deposited at target receptor sites within the lung whilst reducing systemic concentrations and hence the adverse side effects when these drugs are administered orally or intravenously.

Interest in the use of inhaled medications for use in performance horses has grown considerably in recent times, largely due to the introduction of the metred dose inhaler (MDI) (Hoffman 1997) and several coupling appliances including the Equinehaler™, which have been designed specifically for this purpose (Duuvivier, Votion et al. 1997). However, the Australian Rules of Racing (A.R.178B) state that inhaled bronchodilatory agents such as ipratropium bromide (Atrovent®) and inhaled corticosteroid agents such as fluticasone propionate (Flixotide®), are prohibited substances. Although these drugs are commonly administered to horses in race training and a wide array of other performance horse disciplines, detection of these agents in horses is not permitted on race or competition days. Therefore, there was a need to determine the length of time these drugs can be detected post administration of routine therapeutic doses via inhalation therapy.

Objectives
To determine the excretion data and detection times for ipratropium bromide (Atrovent®) following administration of therapeutic doses via an Equinehaler™ using an MDI. To this end, establishment of methods for extraction of parent drugs from equine urine, their detection and quantification by liquid chromatography mass spectrometry (LC/MS), and determination of their excretion profiles were required.

Methodology
Aerosolised ipratropium bromide monohydrate (Atrovent® MDI 20µg/actuation), (Boehringer Ingelheim, Australia Pty Ltd) was administered to six Standardbred (SB) mares at a dose rate of 250 µg twice daily (q12h) for 72 hours (3 days) via an Equinehaler™ delivery device (DLC Australia Pty Ltd, Caboolture). Urine samples were collected via catheterisation at 0, 3, 6, 9, 12, 24, 48, 72 hours following cessation of treatment. Urine samples were divided into two equal aliquots, stored at -20°C and shipped to the Australian Racing Forensic Laboratory, Randwick NSW, for analysis. Collected urine samples were subsequently analysed by liquid chromatography mass spectrometry (LC/MS) to determine the detection parameters.

The most commonly used screening technique for quaternary ammonium drugs in equine urine is by solid phase extraction (SPE) of drugs from the urine and detection of target analytes by LC/MS. For the purposes of this study, a Thermo-Finnigan TSQ Quantum Ultra triple- quadrupole mass spectrometer was used for LC/MS which was equipped with a Surveyor Auto-sampler, degasser and MS pump system (Thermo Finnigan, San Jose, CA, USA). In addition, a single column SPE method was employed as this method is frequently utilised for extraction of many drug classes during routine screening by LC/MS. Samples were analysed in the selective reaction monitoring (SRM) mode using an electro-spray ionisation (ESI) source. Ipratropium transitions monitored were m/z 332 → 166 for quantitation, and m/z 332 → 124 and m/z 332 → 290 as qualifiers. The internal standard pipenzolate transition monitored was m/z 354.2 → 144.2.
Statistical Methods
Data were analysed using Microsoft Excel and GenStat (Version 8.0) and consisted of readings of concentrations of ipratropium bromide taken at eight individual time points over 72 hours. As there were no treatment comparisons to be made, the only analysis that could be performed was to fit an appropriate model to each horse’s data. Moreover, the data were serially correlated.

Results and Discussion
Although the intra-individual variations in urinary concentrations were small, large inter-individual variations in the total amount of ipratropium bromide excreted were observed. Therefore, Gaussian (or normal probability density) curves were fitted to the data where the function for the Gaussian model was:

\[ C_{\text{oncentration}} = \alpha + \left( \beta / \sqrt{2\pi \sigma} \right) \times e^{-0.5(\mu-\mu)^2 / \sigma^2} \]

Where;
\( \alpha \): Approximate concentration of ipratropium at time zero, or, given the nature of the data, the concentration that eventually remains in the urine
\( \beta \): Controls height of the response (thus controls maximum concentration)
\( \mu \): Time the maximum concentration is achieved
\( \sigma \): Spread of concentrations over time.

Separate models were tested, constraining \( \alpha \) to be zero, but little or no gains were made by undertaking this procedure. The fitted models for each horse are shown in Figure 8.

Figure 8: Data and fitted models for each horse, shown on the same scale for comparison.

Gaussian curves were chosen to fit these data because the right hand tail asymptotes to a near zero concentration. However, since the data tailed off at hour nine, there are only four data values to fit a
three-variable (if we set $\alpha = 0$) or a four-variable model. The derived excretion curves (averages) are shown in Figure 9.

![Ipratropium Excretion Curve (Average)](image)

**Figure 9:** Average Excretion of Ipratropium Bromide (Atrovent®) in Equine Urine for each Horse ($n=6$) at each Collection Time (0, 3, 6, 9, 12, 24, 48, 72 hours Post-Administration).

Aerosolised ipratropium bromide (Atrovent®) was assayed up to 72 hours post cessation of drug administration. Peak detection of ipratropium bromide occurred at three hours post-administration followed by a rapid decline in excretion between three hours and six hours post-administration. Using solid phase extraction (SPE) followed by LC/MS, the limit of detection for ipratropium bromide is approximately 50 $\rho$g/mL (0.05 ng/mL). However, the concentration at which ipratropium bromide can be confirmed is approximately 100 $\rho$g/mL (0.1 ng/mL) which is equivalent to six to nine hours post-administration.

**Conclusions**

Results from this study demonstrate that aerosol administration of 250µg of ipratropium bromide (Atrovent®) via Equinehaler™ every 12 hours for three consecutive days can result in positive detection of the drug in urine, with peak concentration detected at three hours post cessation of treatment and declining significantly between six and nine hours post-administration. However, the period of detection stated should not be interpreted as a recommended withholding period.

**Study 3B: Detection of Inhaled Fluticasone Propionate (Flixotide®) in Equine Urine using High Performance Liquid Chromatography (HPLC)**

**Introduction**

Since their introduction almost 30 years ago, inhaled corticosteroids have played an integral role in the treatment of human airway diseases (Hardman and Limbird 1996). Through their action as potent anti-inflammatory agents, these drugs have been used to treat chronic airway inflammatory conditions such as asthma, chronic obstructive pulmonary disease and other inflammatory airway disease of the large and small airways (Hamid, Song et al. 1997); (Laitinen, Laitinen et al. 1992; Lawrence, Wolfe et al. 1997).

Similarly, corticosteroids have become the mainstay treatment for inflammatory conditions of the equine airways including RAO and IAD. Corticosteroids affect all aspects of the inflammatory
response (May 1992) and are therefore the most effective of the anti-inflammatory agents in clinical use. Corticosteroids (e.g. prednisolone) have routinely been administered via the systemic route, where they must be absorbed from the GIT and reach the systemic circulation to be effective in the treatment of respiratory diseases. However, long-term systemic administration of corticosteroids to horses may produce undesirable side-effects such as muscle wasting, hyperglycaemia, polydipsia, polyuria, laminitis and immunosuppression (Eyre, Elmes et al. 1979; Macharg, Bottoms et al. 1985; Cohen and Carter 1992).

To abrogate the disadvantages of systemic administration, other routes of administration such as aerosol delivery via inhalation, have been investigated more recently. The inhalational route of administration provides deposition of high concentrations of drug at target receptor sites within the lung, therefore increasing the biological availability of the administered drug whilst simultaneously reducing systemic concentrations and hence adverse side effects. In addition, the characteristics of the equine airways and patterns of breathing are well suited to inhalation therapy, as the large tidal volume, high flow rate and obligate nasal breathing of the horse encourage aerosol deposition deep within the lung.

Inhaled fluticasone propionate (Flixotide®) has been shown to be beneficial for treatment of chronic airway inflammation in horses, but information is lacking on its efficacy for more acute conditions such as lower airway inflammation in young performance horses. Furthermore the duration of excretion post-administration and the limit of detection (LOD) for this drug are not known. This information would allow more accurate recommendations on drug withdrawal periods following administration of therapeutic doses of fluticasone propionate (Flixotide®).

**Objectives**

To refine the current methods for detection of fluticasone propionate in equine urine and subsequently to provide information on post-administration detection times and limits of detection to racing authorities and treating veterinarians.

However, detection of fluticasone propionate by analysis of biological fluids such as blood and urine represent a significant analytical challenge due to the potency of fluticasone propionate, the low concentrations of drug absorbed following inhalation, and the fact that the drug undergoes extensive first-pass hepatic metabolism into its inactive 17-carboxylic acid derivative.

**Methodology**

Aerosolised fluticasone propionate (Flixotide® MDI 250µg/actuation), (Glaxo Smith Kline, Australia Pty Ltd) was administered at a dose rate of 1,000 µg twice daily (q12h) to six (n=6) standardbred (SB) mares via an Equinehaler™ delivery device (DLC Australia Pty Ltd, Caboolture). Horses were administered the drug for 72 hours prior to the commencement of urine sample collection.

Urine samples were collected via catheterisation at 0, 3, 6, 9, 12, 24, 48, 72 hours following cessation of treatment. Urine samples were divided into two equal aliquots, stored at -20C and shipped to the Australian Racing Forensic Laboratory, Randwick NSW, for analysis.

LCMS analysis was performed using a Thermo Finnigan TSQ Quantum Ultra triple-quadrapole mass spectrometer equipped with a Surveyor Auto-sampler, degasser and MS pump system (San Jose, CA, USA).

Fluticasone propionate is primarily excreted as a carboxylic acid derivative (Harding 1990), which requires an initial base hydrolysis procedure before analysis via HPLC. A volume of 10µL of the extracted sample was loaded onto a Waters Xterra MS C18, 2.5µm; 50 x 2.1mm column (Milford, MA, USA). The chromatographic solvent system comprised a gradient elution using ammonium acetate 10mM pH4 (A) and methanol (B). Initial conditions involved 100% A changed with a linear gradient to 100% B over 3 minutes, held for a further 2 minutes before returning to initial conditions.
in 1 minute. In addition, spiked urine samples at concentrations of 100pg/ml, 500pg/ml, 1ng/ml, 5ng/ml, 10ng/ml and 50ng/ml were extracted in order to determine the limit of detection for this method for fluticasone propionate.

Samples were analysed in the selective reaction monitoring (SRM) mode using an Atmospheric Pressure Chemical Ionisation (APCI) source run in negative ion mode. For confirmatory purposes, three individual SRM experiments were performed. Fluticasone transitions monitored were m/z 451 → 395, m/z 332 → 329 and m/z 451 → 377.

The limit of detection (LOD) for the method was defined as the concentration giving a signal to noise ratio of three. For fluticasone propionate this was determined to be approximately 5ng/mL.

**Results and Discussion**

When the carboxy metabolite of fluticasone propionate was monitored using only the 451 to 395 transition it was determined that the lowest detectable level was 500 pg/mL (see Figure 10). This transition may be used for screening purposes, but positive confirmation of the presence of the drug in urine requires identification of two further transitions (332 to 329 and 451 to 377). To detect these additional transitions further extraction of a larger volume of urine was required. Therefore, based on extraction of 10 mL of urine, the limit of detection using all three SRM transitions was determined to be approximately 5ng/mL (See Figure 11).

**Conclusions**

Results from this study demonstrate that administration of 1,000µg of fluticasone propionate (Flixotide®) every to 12 hours for 3 days to horses via an Equinehaler™ can be positively detected in equine urine. This study ascertained the LOD for positive confirmatory purposes as detected by LCMS to be 5 ng/mL, with a concentration of 500 pg/mL being sufficient for preliminary screening purposes. Although no metabolites of fluticasone propionate could be detected nine hours after cessation of treatment in the six horses included in this study, it should be remembered that inter-horse variability in metabolism and therefore excretion of the drug is highly variable and therefore, the time at which samples were assessed to have negative detection of fluticasone propionate in the current experiment (>9 hours post-withdrawal) should not be extrapolated to the wider horse population.

Finally, the Australian Rules of Racing (A.R.178E) prohibit the administration of any medicinal substance to horses on race days. Therefore, the period of detection stated should not be interpreted as a recommended withholding period.
Figure 10: Urine spiked with 500 pg/mL fluticasone propionate demonstrating one transition peak, which is adequate for screening purposes.

Figure 11: Urine spiked with 5 ng/mL fluticasone propionate demonstrating all three SRM transitions which are required for positive identification of the carboxylic acid derivative of fluticasone propionate.
STUDY 4: Evaluation of the Efficacy of Aerosol Therapy for Resolution of Pulmonary Neutrophilia Associated with Lower Airway Inflammation in Young Performance Horses using RCT

Introduction
The respiratory system is considered to be one for the limiting factors for maximal exercise in horses. Sub-clinical or moderate pulmonary dysfunction, may lead to a reduction in aerobic metabolism and poor performance (Wagner, Gillespie et al. 1989; Art, Anderson et al. 1990). Inflammation of the airways is characterized by cough (Christley, Hodgson et al. 2001), increased tracheobronchial mucus (Robinson, Berney et al. 2003) and presence of elevated numbers of inflammatory cells, specifically neutrophils in the large airways. This latter phenomenon, referred to in our studies as LAI has been estimated to occur in ~25-50% of Thoroughbred and Standardbred racehorses at some stage during their preparation for racing. This inflammatory response has been associated with impaired performance and interruption to training (Burrell 1985; Macnamara, Bauer et al. 1990; Sweeney, Humber et al. 1992; Malikides 2003).

It has been ascertained that long term amelioration of LAI requires minimising exposure of horses to pro-inflammatory inhaled environmental agents such as dust associated endotoxin. However, given the difficulty or ensuring total absence of these environmental stimuli, additional benefits may be gained via use of pharmaceutical agents. To date treatment modalities for LAI have been commonly based empirically and as such are speculative. For example in one survey it was reported that antibiotics are likely to be over-prescribed by veterinarians when treating horses with cough and other signs consistent with LAI (Christley 1999). However, few field based clinical trials have been conducted on race horses comparing the individual efficacy of available pharmaceuticals, in particular, inhalational aerosol preparations on the reduction of LAI.

Objective
To determine whether administration of therapeutic doses of inhaled pharmaceuticals (stand alone steroid therapy) and combinations therapy (bronchodilator and steroid therapy) via an Equinehaler™ provides benefit in the absence of environmental control measures for the resolution of cough, pulmonary neutrophilia and increased tracheobronchial mucus associated with LAI in young performance horses.

Methodology
RCT was conducted on young racing horses (2.7±0.7 years) housed at Sydney metropolitan race stables over 16 months. Nine stables (training establishments) accommodating Thoroughbred and Standardbred racing horses participated in the study. Horses were selected in the trial if they fit the inclusion criteria; horses were 2-4 years old and had coughed > 4 times in a 10 minute time frame during or immediately following exercise (see Christley, 1999). One of the authors (PJS) was in regular contact with trainers and once a horse was identified as possible candidate for inclusion a routine investigative process was undertaken.

Horses identified as possible candidates for the RCT were first assessed via endoscopy to determine their respiratory tract health (see Figure 12). A ‘mucus score’ for both the upper and lower respiratory tract was assigned to each horse as per Robinson (2003). A tracheal aspirate (TA) sample was collected via a guarded catheter for cytological and bacteriological evaluation. Horses were included in the current study if the cellular population was composed of >20% neutrophils but <5% eosinophils.
Horses were excluded from this study if the bacteriological population of the fluid retrieved by TA had $10^3 > \text{cfu/mL}$ as these horses represented a different population from the one under investigation in this study.

**Figure 12:** Use of a videoendoscope for visualisation of the respiratory tract for diagnostic purposes.

Following confirmation of the presence of LAI, horses (‘cases’) commenced treatment within 24 hours and were randomly assigned to:

- **Treatment 1** [steroid therapy]: Flixotide® (fluticasone propionate) -1,000µg q12h; n=21
- **Treatment 2** [combination therapy]: Atrovent® (ipratropium bromide [bronchodilator] 500µg q/12h followed by Flixotide® - 1,000µg q12h; n=20).

Horses were administered these pharmaceutical agents for 14 consecutive days via a metered dose inhaler (MDI) with an Equinehaler™ used as a coupling device. Subsequent endoscopy, mucus scoring, bacteriological and cytological evaluation of TA samples were performed on Day 7 and Day 14, i.e., at cessation of treatment. The results of differential TA cytology from Day 0, Day 7 and Day 14 of treatment were statistically compared.

**Recruitment of Participating Trainers & Veterinarians**

At the commencement of this study, each trainer recruited signed a Participants’ Consent Form. In order to be informed about the trial all participating trainers were provided with an outline of the study detailing the aims and objectives of the study, and a simplified schematic representation of the study design, duration and procedures (see Figure 13). As a result of relationships established during trials conducted by this research group over the past 15 years the majority of trainers and veterinarians approached were willing to participate. The compliance of trainers in particular, was integral to the success of the current study, and their cooperation and ease of communication for the duration of this study is to be lauded.

The stables chosen for inclusion in this study were selected on the basis of several criteria. First, they were all located in a defined geographical area of the Sydney basin. Second these establishments included what is considered a representative sample population of young Thoroughbred and Standardbred racehorses. Third, each of the trainers involved was aware of the significance of LAI and as such was willing to participate in the trial. Seven Thoroughbred training stables participated in the study, being recruited from two major metropolitan racetracks in Sydney; Warwick Farm and Rosehill. The two Standardbred training stables were located near Camden, southwest of Sydney.
A number of key points were repeatedly stressed to trainers to ensure there was minimisation of misunderstandings as to which horses could be considered as ‘cases’ and therefore incorporated in the current study. These points included:

- A horse must experience $\geq 4$ coughing episodes within ten minutes during or immediately following work,
- The animal must be in training,
- The horse must be housed in a stable and have been on the same bedding material type for $\geq 2$ weeks, and be able to remain on that bedding type for the duration of the 14 day study period,
- The horse must not have been transported within 48 hrs of the examination,
- The horse must not be receiving or will receive any other medications during the treatment, or have a concurrent illness,
- Endoscopy was to be performed and samples collected on three occasions; Days 0, 7 and 14. As a result assistance may be required by stable staff to restrain the horse during each visit.

All endoscopic examinations, TA cytology, bacterial culture and supply of pharmaceuticals were free of charge to the owner and trainer. All results, when obtained and confirmed, were available for discussion with the trainers and treating veterinarians.

In addition, in accordance with the Australian Rules of Racing it was emphasised to trainers that all pharmaceutical agents used in the trial had a 72 hour withholding period prior to racing. Finally, it was specified that no drugs would be left on the premises and that one of the author (PJS) would be the sole administrator of drugs for the duration of the study.

**Schematic Flow of Events**

A general schematic of the RCT is depicted in Figure 13. Data was collected from eligible horses at designated Day 0, again at Days 7 and 14 – the latter being the day treatment was discontinued. Resolution of lower airway inflammation was assigned as the primary study outcome.

This design allowed exploration of a large number of variables, their interactions and their association with the study outcome. Furthermore, data was collected at these time points as results from earlier studies indicated that two weeks treatment were considered necessary to allow the effects of treatment to be consistently exerted (see Study 2). Additionally, 14 days was not considered excessively long in terms of the commitments required by the trainer. We also estimated that by using the weekly sampling intervals this would allow adequate resolution of any direct effect of the previous TA sampling whilst also avoiding frequent impositions on trainers. Of course we recognise that more frequent sampling over a longer study period may have allowed more detailed analysis of the temporal effects of extraneous confounders on the resolution of airway inflammation. However, we concluded that the final experimental design was an appropriate compromise between the wishes of the investigators, tolerance of trainers, yet provided the opportunity to provide practical, applicable results.

**Data Collection**

One of the investigators (PJS) was solely responsible for all data collection, which was hand written onto prepared information sheets at the time horses were examined and subsequently transferred to computerised databases. At the time of initial examination a number of specific questions were asked to identify prior and current problems regarding the horse. Questions of a subjective nature to the trainers were kept to a minimum to reduce non-differential measurement bias resulting from poor recall of current and past events. However, a variety of additional non-subjective data was also recorded. In addition, to ensure an adequate degree of validity, leading questions were avoided and some questions were repeated in an alternate way to determine if the same response was elicited.
Figure 13: Schematic flow diagram outlining the order of events for the RCT.
Statistical Methods
Data were recorded in computer databases (Microsoft Excel, MS Office 2000, Microsoft Corporation, USA). The entire data set was entered twice and approximately 20% of randomly selected horses were crosschecked with original records for internal consistency. Resultant outlying values for variables were reviewed and corrected due to entry error. Categorical variables were also reviewed to ensure biological plausibility of categories.

The study was analysed using a variety of statistical methods. In general the main analyses involved multivariable modelling techniques facilitating assessment of associations between a large number of variables and a selected outcome. All analyses were performed using GenStat® (Version 8 and 9) and Microsoft Excel. Due to the nature of the data, a Repeated Measures analysis in REML® was used to ascertain change in percent neutrophils over time. Three different correlation structures (Uniform correlation with equal variances, Uniform correlation with unequal variances, and Unstructured correlation) were tested on each time point (Day 0, Day 7, Day 14) to ascertain the most appropriate model. In addition, Linear Mixed Model analyses were performed and as an approximation method, multivariate split-plot-in-time analyses were performed, and correlations constructed using Sums of Squares and Product matrices.

Primary variables of interest included the assigned pharmaceutical treatment, age, gender, breed and bedding material the horse was stabled on. Trainer effects were also of interest. In addition, the study involved the effect over three time periods, so these were also included in the analyses.

Results and Discussion

Neutrophil percentage and correlation with lower respiratory tract mucus score
For the neutrophil percentages versus LRT mucus scores, there was a weak linear association with \( r = 0.443 \) at the Treatment level. Similarly there was a weak correlation between these scores \( r = 0.439 \) at the Day level. However, with this latter correlation (see Figure 14) there was a reduction in the percentage of neutrophils in TA with increasing treatment duration.

![Figure 14: Period (day) means of neutrophils and LRT mucus scores](image-url)
Correlations of upper and lower respiratory tract mucus scores
There was no linear association found for URT versus LRT mucus scores at the Treatment level ($r = 0.173$), and at the Day level ($r = 0.245$). This is shown graphically in Figures 15 and 16.

**Figure 15:** Treatment means of LRT and URT mucus scores

**Figure 16:** Period (Day) means of LRT and URT mucus scores

In addition, the results revealed no demonstrable difference in the efficacy of the two treatments in terms of their effect on neutrophil percentages ($p=0.074$) (see Figure 17). Both treatments resulted in a progressive decrease in percentage of neutrophils with increasing duration of therapy (Day 7-0, $p=0.001$; Day 14-7, $p=0.001$).
In addition to determining the effect of, and differences between, each of the treatments the data were examined for other associations. These included the effect of trainer, horse age, horse gender, horse breed and bedding material. All variables were examined for confounding effects. Of these, only trainer had any influence on the results, with there being an interaction between trainer and TA neutrophil percentages.

In summary the results of this study show that steroidal anti-inflammatory inhalation therapy alone was equally efficacious as a combination of the steroidal agent and a bronchodilator in ameliorating LAI. In addition, there was an influence of trainer on neutrophil percentages in TAs of horses in racetraining.

**Conclusions**

There is an abundance of anecdotal information regarding the purported efficacy of treatments for LAI. Much of this comes from overseas where imposts on respiratory health of horses differ to those occurring in Australia. Overall, there is paucity of data from controlled clinical trials and the specific application of this information to the Australian performance horse industries. It is therefore not surprising that there are many non-specific, empirically based treatment regimens for LAI currently in use in Australia. This prompted us to undertake the study reported in this section.

In the current study we chose to evaluate the efficacy of an aerosolised steroid as one of the treatment regimens as there is strong evidence to indicate these drugs reduce lower airway inflammation in horses with a variety of respiratory diseases. In addition, we investigated the combination of the inhaled steroid with a drug inducing bronchodilation as previous studies in horses with chronic respiratory disease have shown that the efficacy of inhaled steroids is augmented if bronchodilation is invoked prior to inhalation of the steroid. It is postulated that this dilation of the lower airways is requisite for optimum deposition of the steroid in peripheral airways. However, although this combination therapy has been shown to be effective in chronic respiratory diseases such as ‘heaves’, where there is a well demonstrated relationship between the disease, bronchoconstriction and airway inflammation, such a relationship was not clear for LAI in young performance horses. In addition, current data suggests that inflammation may be the key factor in respiratory dysfunction in LAI and bronchoconstriction may not be a significant feature of this syndrome. Therefore, we undertook the current trial to further elucidate the effectiveness of both an inhaled steroid as well as the steroid in
combination with a bronchodilatory agent, to determine if either treatment regimen is effective for
treatment of this syndrome.

Results from the RCT conducted in the current study demonstrated that both treatments were assessed
as being equally efficacious. In other words the use of a bronchodilator concurrently with a
corticosteroid did not provide better efficacy than steroidal intervention alone. Therefore, we
recommend that steroidal aerosol therapy with the drugs and doses used in the current study may be an
effective and non-invasive way of treating LAI. Such therapy should always be amalgamated with
apposite environmental management; for example, reduction in exposure to dust associated endotoxin
and other improvements in air quality. The lack of indication for use of a bronchodilator has several
positive implications. One is that treatment will be much less time consuming and labour intensive
(one drug vs. two) and will result in a more economical, yet efficacious treatment strategy.

Concerns may be raised regarding the potential for increased likelihood of respiratory infections in
horses following use of inhaled steroids. This is to be expected as steroids are immunosuppressive
drugs. During the early years of treatment of asthmatic human patients with inhaled corticosteroids,
this same concern was expressed. However, this apprehension has proven to have no clinical
foundation (Brogden, Heel et al. 1984), and even in immunocompromised human subject, the
incidence, severity and duration of viral or bacterial respiratory infections is not increased by inhaled
steroid treatment (Toogood 1990). Although no adverse effects were observed in horses in the current
trial, or under other clinical settings where inhaled steroids have been prescribed in horses, caution
may still be warranted until large prospective studies are conducted.

In conclusion, based on the current clinical trial, inhaled steroids should remain as one of the
cornerstones of therapy for both chronic, debilitating respiratory ailments such ‘heaves’ as well as
more acute respiratory diseases in equine athletes including LAI.
Recommendations

Improved Environmental Management

The environmental control of respiratory disease in stabled horses requires maintenance of breathing zone respirable challenge below a minimal threshold limiting value (TLV). Therefore appropriate management practices are required to ensure effective control and prevention of lower respiratory tract irritation with resultant inflammation. Previous studies conducted elsewhere in the world report clinical and functional improvements in respiratory health as a direct result of implementation of environmental control measures (Thomson and McPherson 1984; Jackson, Berney et al. 2000). This was especially relevant for horses housed on straw and fed hay as the main feed-stuff (McGorum, Ellison et al. 1998).

The concentration of dust within stables fluctuates and is often increased by general management strategies. Highest concentrations have been associated with animal and human activity (Debilquy, Nicks et al. 1991). In the few studies where various stable activities have been compared, particle counts recorded during feeding and cleaning were up to five times greater than when horses were resting (Crichlow, Yoshida et al. 1980; Webster, Clarke et al. 1987; Woods, Robinson et al. 1993). Not surprisingly a balance needs to be achieved in order to minimise dust concentrations. So, although on one hand the cleaning of aisles and stable boxes generates of dust, these practices are critical to limiting overall exposure to dust. For example, frequent replacement of bedding, especially when soiled or wet, aides to reduce the production rate of airborne particles (Clarke and Madelin 1987). Thus, removal and replacement of bedding although producing dust is less likely to result in persisting relatively high concentrations of dust as will be the case if bedding becomes soiled.

Bedding and feed choices are critical to ensure appropriate diminution of the particulate load within the stable environment. In conventional horse management systems where horses are kept indoors, bedded on straw and fed hay there is resultant higher dust exposure than occurs when wood shavings and pelleted feeds are used or if the horses are kept at pasture (Woods, Robinson et al. 1993; McGorum, Ellison et al. 1998; Holcombe, Jackson et al. 2001). Similarly in other studies when straw bedding was evaluated against shredded paper and sawust, straw was found to produce substantially higher concentrations of total and respirable airborne endotoxin (Vandenput, Istasse et al. 1997; Tanner, Swinker et al. 1998; Ward, Wohlt et al. 2001).

Strategies for the reduction of feed related particulate matter and endotoxin include soaking hay (for at least 30 minutes) prior to consumption, which reduces respirable concentrations without excessive nutrient loss (Moore-Colyer 1996). Although pellets are used as part of a dust reduction strategy, if they are friable and feed handling is rough, concentrations of generated dust may be high (Cargill, Skirrow et al. 1995). Tallow, vegetable and soybean oils have also been successfully used in the pig and poultry industries in addition to pelleted feed (Dawson 1990; Heber and Martin 1998), with reductions in dust concentrations up to 85% reported (Li, Owen et al. 1996).

Ventilation is a major factor potentially mitigating against induction of LAI by virtue or improved rate of air exchange, which has a positive influence on the air quality in stables (Webster, Clarke et al. 1987). Horses housed in stables where ventilation is insufficient are exposed to increased concentrations of airborne particles and may subsequently develop increased tracheal muco-pus (Clarke and Madelin 1987) and lower airway inflammation (Holcombe, Jackson et al. 2001).

However, under a variety of circumstances natural ventilation of stables cannot be relied upon to provide satisfactory air quality (Dunlea and Dodd 1996). For example, ‘American type’ barns, which are naturally ventilated buildings with smaller openings, generally provide inadequate ventilation rates (Wathes 1994). On the other hand, a well designed box with the upper door open is more likely to meet the needs of the horse and even in still air conditions, the ventilation rate is reported to always...
exceed the minimum of four air changes/hour required (Webster, Clarke et al. 1987). In other intensive livestock production systems fogging, showering and misting sheds with water have been employed with the aim to reduce dust. Although some dramatic effects have been reported in terms of reducing total dust, little effect on respirable dust has been reported (Rhyr-Anderson 1990) and these techniques would appear to have little use in equine facilities.

A minimum **dust management regimen** is crucial for the effective control and prevention of LAI in horses. Thus, as discussed above it is essential that care is exercised when selecting bedding whilst also ensuring the stable is adequately ventilated. Selection of the least dusty feedstuffs also contributes to an overall dust management regimen. It is also important, if possible, to store feed and bedding material away from the stable environment either in another building or in a room with an independent airspace and ventilation system (Woods, Robinson et al. 1993). Additionally, cleaning stables while horses are *in situ* is not recommended, as the concentration of airborne particles will be highest while bedding is being removed/changed.

**Treatment of Airway Inflammation using Aerosolised Medications**

Pharmacological treatment of airway inflammation in horses has traditionally consisted of bronchodilatory and anti-inflammatory drugs (Derksen 1991). In recent times, the therapeutic emphasis for asthma in human patients has shifted from bronchodilator therapy when symptoms occur to daily anti-inflammatory therapy to assist in preventing episodes of airway obstruction (Varner and Busse 1996). Similarly, inflammation is now recognised as the underlying pathophysiological process in all cases of bronchoconstriction in the horse. For example in ‘heaves’, a chronic irreversible disease of older horses most commonly encountered in the Northern Hemisphere, repeated anti-inflammatory therapy can eliminate the need for bronchodilator therapy (Rush 2001).

Interest in inhaled medications for equine use has grown considerably in recent years. However, it is important to note that the efficacy of therapeutic aerosols is a function of the dose of aerosol deposited in the lower respiratory tract and the distribution of the drug within the airways (Derksen and Robinson 1998). Depending on the administration device, the relative proportions of delivered drug to the small airways may vary. Therefore, when determining dosing schedules, the clinician should carefully consider the performance of the delivery device used. This is why we used the same coupling device (Equinehaler®) for all experiments described in this report.

One group of agents that have received significant attention for treatment of lower airway inflammation are inhaled corticosteroids. The corticosteroid fluticasone propionate (Flixotide®) has particularly received considerable attention as it is the most potent drug in this group, has the longest pulmonary residence time, and has the least potential for induction of adrenal suppression (Varner and Busse 1996). Various studies in horses have also shown that inhaled fluticasone is beneficial in assisting the resolution of airway inflammation (Viel, Staempfli et al. 1999; Giguere, Viel et al. 2002; Couetil, Chilcoat et al. 2005) and improving pulmonary function in horses with clinical ‘heaves’. Furthermore, if inhalation therapy is combined with environmental modification even greater improvements can be achieved. For example, in one study the clinical score and pulmonary function of ‘heavey’ horses were improved significantly within two weeks after horses were placed in a low-dust environment and received treatment with fluticasone propionate (Couetil, Chilcoat et al. 2005).

Studies by our group and others have shown that LAI is as a common disorder of young performance horses worldwide, including in Australia (Malikides and Hodgson 2003). This syndrome, is different to the more chronic lower respiratory tract condition of horses called ‘heaves’ as the inflammation observed in LAI occurs without the permanent, long term lung changes that are present in horses with ‘heaves’. However, although the endstage pathological process may differ between LAI and ‘heaves’, the presence of inflammation is central to both syndromes. Therefore, administration of aerosolised corticosteroids, which had been used successfully for many years to treat ‘heaves’, has obvious
implications for dampening the inflammation observed in LAI. The studies we present in this report demonstrate that inhaled fluticasone has excellent effects in reducing lower airway inflammation in younger performance horses under experimental and field conditions. As such, we are confident in recommending this agent for use in horses with LAI, particularly if combined with improvement of the housing environment. Finally, although inhaled corticosteroid therapy is associated with a low risk of systemic effects compared to oral or parenteral administration, systemic absorption of corticosteroids still occurs following inhalation (Goldberg, Algur et al. 1996; Yiiallouros, Milner et al. 1997; Mollmann, Wagner et al. 2001). Therefore, we recommend that the safety of prolonged (greater than 2 weeks) administration of fluticasone in horses should be addressed in a larger prospective study. In our current study, where treatment persisted for 14 days, we recognised no adverse effects, but no attempts were made to measure serum cortisol concentrations or the level of systemic absorption of inhaled fluticasone propionate.

In conclusion, we recommend corticosteroid therapy, administered via aerosol using a recognised coupling device, as part of an appropriate therapeutic approach in horses with LAI. Although we have demonstrated that aerosolised corticosteroids are effective for the reduction of LAI in racehorses, we suggest that improvement of air quality is also essential. This latter arm of the treatment is to ensure that the inciting environmental stimulus is removed such that horses are not continually exposed to high concentrations of pro-inflammatory mediators through the air they breathe.
Publications & Presentations

2007
P. Spendlove, J. Hodgson, D. Hodgson, N. Malikides. *Potential Exposure of Horses to Total and Respirable Particle Endotoxin Concentrations Generated by Specific Feed and Bedding Materials.* (Accepted Equine Veterinary Journal).


2006
P. Spendlove, J. Hodgson, D. Hodgson, N. Malikides. *Potential Exposure of Horses to Total and Respirable Particle Endotoxin Concentrations Generated by Specific Feed and Bedding Materials.*

*Abstract and Oral Presentation* Australian Equine Science Symposium (AESS), June 2006, Gold Coast Australia.


2005


2004

References


Bayly, W. M. (2005). Inflammatory Airway Disease. 11th annual meeting of the Italian assec equine veterinarians (SIVE), Pisa, Italy.

Bayly, W. M. (2005). Inhalation therapy and respiratory disease. 11th annual meeting of the Italian assec equine veterinarians (SIVE), Pisa, Italy.


Poster presentation


Robinson, N. E. Effect of respiratory disease on the response to exercise. Bain Fallon, need to find out year.


Rylander, R. (2001). The role of endotoxin and (1-->3)-B-D-Glucan as synergistic agents in lung inflammation and allergy. WEAS.


