Inflammatory Airway Disease in Young Thoroughbred Racehorses

A report for the Rural Industries Research and Development Corporation

By N. Malikides and J. L. Hodgson

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Foreword

Despite the size of the Australian racing industry and the significant wastage that occurs as a result of respiratory disease, only limited research has been conducted evaluating the occurrence of and risk factors for inflammatory airway disease in racing thoroughbreds in this country. Furthermore, although there is now good scientific information available about infectious agents associated with lower airway inflammation in racehorses, little is known about non-infectious stable management and dust related agents that may also be involved.

This publication summarises the findings of a three-year study investigating inflammatory airway disease in two to three year old racing thoroughbreds. Chapter 1 reviews relevant literature, specifically examining, in the context of the unique environment in which young racehorses are placed, how infectious and non-infectious particles enter the lower airways, how horses defend themselves from these agents, and what inflammatory effects might ensue and why. The next three chapters outline the design and results of an epidemiological study in which the primary aims were to determine the incidence of inflammatory airway disease (IAD) in young thoroughbred racehorses entering racetrack stables for training and to evaluate principally non-infectious management and environmental risk factors for this important respiratory disease.

The information in this report is the first large longitudinal study investigating lower airway inflammation in young racehorses and the first to evaluate the role of inhaled dust endotoxin and (1→3)-β-D-glucan. The results provide compelling evidence that current stable management practices and a reliance on natural ventilation is insufficient to prevent IAD and that there is an urgent need to rethink current strategies aimed at reducing airborne particle burdens and preventing the high incidence of this disorder in racehorses. This project has involved national and international collaboration and has expanded and improved upon previous models of research, also funded by RIRDC.

This project was funded from industry revenue, which is matched by funds provided by the Federal Government.

This report is an addition to RIRDC’s diverse range of over 1000 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.

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Simon Hearn
Managing Director
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Abbreviations

APC  Antigen presenting cell
BAL  Bronchoalveolar lavage
BALT Bronchial associated lymphoid tissue
CI  Confidence interval
cm  Centimetre
CO₂ Carbon dioxide
cfu Colony forming units
EHV Equine herpes virus
EIPH Exercise-induced pulmonary haemorrhage
ERV Equine rhinitis virus
EU Endotoxin units
IAD Inflammatory airway disease
Ig Immunoglobulin
IR Incidence rate
km Kilometre
L Litres
LBP Lipopolysaccharide binding protein
LPS Lipopolysaccharide
LRT Lower respiratory tract
LAL *(Limulus*) amoebocyte lysate
m Metre
m² Square metres
m³ Metre cubed
µm Micrometre
mL Millilitre
ng Nanogram
NH₃ Ammonia
PAS Personal air sampler
PG Prostaglandin
PLH Pharyngeal lymphoid hyperplasia
ppm Parts per million
OR Odds ratio
RAO Recurrent airway obstruction
rpm Revolutions per minute
TA Tracheal aspirate
Tc Cytotoxic T cells
Ts Suppressor T cells
Th T helper
TLV Threshold limiting value
URT Upper respiratory tract
# Contents

Foreword ........................................................................................................................................ iii  
Acknowledgments .......................................................................................................................... iv  
Abbreviations ................................................................................................................................ v  
Contents ......................................................................................................................................... vi  
Executive Summary ..................................................................................................................... viii  
1. What is Currently Known About Inflammatory Airway Disease in Young Racehorses?........ 1  
   1.1 Introduction ........................................................................................................................... 1  
   1.2 Structure and Function .......................................................................................................... 2  
   1.3. Airway Defences and Inflammation .................................................................................... 5  
   1.4. Causes of Inflammation ..................................................................................................... 13  
2. General Introduction and Materials and Methods .................................................................... 26  
   2.1 General Introduction ........................................................................................................... 26  
   2.2 Study Design ....................................................................................................................... 28  
   2.3 Data Collection ................................................................................................................... 31  
   2.4 Sample Collection ............................................................................................................... 31  
   2.5 Statistical Analysis .............................................................................................................. 34  
3. Results ....................................................................................................................................... 36  
   3.1 Descriptive Statistics ........................................................................................................... 36  
   3.2 Results of All Data .............................................................................................................. 46  
4. Discussion and Conclusions ..................................................................................................... 52  
   4.1 Study Design Issues, Bias and Limitations ......................................................................... 52  
   4.2 Descriptive Information ....................................................................................................... 53  
   4.3 Overview of Analyses for Cohort and Case-Control Studies ............................................. 57  
   4.4 Key Implications ................................................................................................................. 62  
5. References ................................................................................................................................. 63
Executive Summary

Inflammatory respiratory disease continues to be a serious cause of wastage to the performance horse industries in Australia and overseas. Substantial losses are incurred from inability to train horses effectively, from costs of veterinary care and medication, and decreased earning potential. In addition, up to 33% of young racehorses in training can have lower airway inflammation without demonstrating any clinical signs of respiratory disease, emphasising the serious welfare implications of this important respiratory disorder.

Recently, studies have demonstrated the significance of specific bacteria and their association with neutrophilic inflammatory airway disease (IAD) in young racehorses, and have highlighted the lack of association with common equine respiratory viruses. However, these studies also underlined the importance of non-infectious environmental factors in producing IAD. Young racehorses housed in a range of stable conditions potentially are exposed to high burdens of aerosolised non-infectious dusts and gases in an almost continuous and cumulative manner. Spending most of their time (up to 22 hours) in looseboxes, racehorses are exposed to variably large burdens of dusts from bedding, feed, loosebox materials, and sources outside the loosebox such as from swept laneways, stable corridors and human and mechanical activity. Endotoxin, a major pro-inflammatory glycolipid component of Gram-negative bacterial cell walls, is one agent that is ubiquitously present in airborne dusts and, as in humans, may play an critical role in the genesis of IAD in young racehorses. In addition, along with the potential for exposure to high dust burdens, racehorses are placed under repeated stresses from transport, sales, variably intense training/racing regimens, intermingling, and housing in frequently poorly ventilated stables. A major outcome of these stressful events is likely to be an increased susceptibility to inhaled agents occurring as a result of corticosteroid-induced impairment in airway defences.

Under these conditions, many young racehorses may therefore be prone to development of lower airway inflammation, which may progress and be complicated by bacterial colonisation, given perpetuation of “stresses” and/or continued exposure to airborne agents. However, the relative contribution of environmental and stable factors, and specific non-infectious dust and gaseous agents over time (longitudinally), to development of IAD in young racehorses housed in stables in Sydney is unknown. In addition, the proportion of racehorses that develop IAD when housed in racetrack stables has not yet been determined under Australian conditions. Consequently, the major aims of the research presented in this report was to determine the incidence of IAD in young Thoroughbred racehorses, investigate as many non-infectious stable management and environmental risk factors for IAD, and to determine the concentrations of specific dust toxins (endotoxin and (1→3)-β-D-glucan) in stable environments and their association with IAD.

A cohort (or longitudinal) study, was designed and carried out between March 2000 and December 2001 using a cohort of 2 and 3-year-old thoroughbred racehorses drawn from a geographically well-defined metropolitan area in Sydney. All eligible, healthy 2 and 3-year-old racehorses that arrived at racetrack stables for training were followed for 4 weeks during which time historical, physical examination, stable management and meteorological information was gathered. In addition, upper and lower respiratory tract endoscopic examination was performed on all horses. From each horse, tracheal aspirate (TA) samples were collected, using a sterile guarded catheter, for cytological and bacteriological analysis, as were blood samples for serological analysis (to detect antibody responses to common respiratory viruses). The same data was gathered from every horse on 3 occasions (Day 0, 14 and 28).
It was found that more than a third of 2 and 3-year-old thoroughbred racehorses entering stables for training already had some form of lower airway inflammation (in which neutrophils or eosinophils or both predominated in TA samples). This most likely reflects exposure to airborne inflammogenic or allergenic agents, or (specifically in the case of eosinophilic IAD) perhaps irregular anthelmintic treatments at pre-training establishments prior to transport to training stables.

Of the racehorses that were IAD free upon arrival to racetrack stables, it was estimated that just over 40% developed neutrophilic lower airway inflammation within the first 2 weeks of stabling. Although in half of these horses IAD resolved during the next 2 weeks, this cumulative incidence of IAD nevertheless is very high and has important implications regarding possible progression of inflammation to clinical disease, decreased training potential and welfare concerns.

Descriptive information on relevant risk factors suggested that hay-type fed (straw-like versus lucerne or clover), and ventilation quality (as determined by window(s) open in the loosebox and carbon dioxide concentrations in boxes), was the most important determinants of neutrophilic IAD in racehorses. In addition, it was found that single endoscopic examinations of proximal lower airways and identification of minor tracheal discharge is unreliable as a means to determine presence of neutrophilic IAD. Finally, no horses in this study were infected with equine influenza virus although most had previously been exposed to Equine Herpes Virus-1 and -4 or recently been infected with Equine Rhinitis Virus A and B. When all relevant variables were examined together, a number of significant risk factors were identified for neutrophilic IAD in young thoroughbred racehorses. Young racehorses were significantly less likely to develop neutrophilic IAD if housed in looseboxes with at least one open window or if all four walls were open to constant ventilation, after controlling for other confounding risk factors. This result emphasises the importance of stable and individual box ventilation in preventing lower airway inflammation. In addition, young racehorses were almost 3-4 times more likely to develop IAD if exposed on average to greater than 5.7 mm/2weeks of evaporation. Although reasons are unclear, this result may reflect levels of humidity, ambient temperature and wind, each of which may contribute to detrimental effects on respiratory defences and airborne dust levels in stables and looseboxes from bedding and feed. Finally, sex, trainer, stable area, factors related to current training, bedding type, quality and age, other stable management factors and infection with common respiratory viruses were not significantly associated with development of neutrophilic IAD in this population of young racehorses.

In order to determine the association between lower airway inflammation and inhaled endotoxin and (1→3)-β-D-glucan found in airborne dust in stables, a case-control study was nested within the cohort study. Dust samples, for determination of endotoxin and (1→3)-β-D-glucan concentrations, and ammonia and carbon dioxide samples were collected only from selected cases and controls. A horse was classified as a case if cytological examination of smears from a TA sample collected either on Day 14 or Day 28 demonstrated > 20% neutrophils (and ≤ 5% eosinophils) on differential count of at least 300 inflammatory cells. In contrast, controls were horses with ≤ 20% neutrophils and ≤ 5% eosinophils on cytological examination of smears from a TA sample.

It was found that a significant linear (or exposure-response) relationship existed between the average percentage of neutrophils in lower airways and exposure to high (>4-5ng/m³) respirable endotoxin concentrations in breathing zone dust. In addition, young racehorses that became cases (as defined) and developed neutrophilic IAD were approximately 4 times more likely to have been exposed to either low (<1.2ng/m³) or high (>4.2ng/m³) concentrations of endotoxin. These associations controlled for the possible confounding effects of many other variables, highlighting the importance of endotoxin alone in the genesis of neutrophilic lower airway inflammation. The effect of endotoxin was likely the result of activation of macrophages and epithelial cells in airways and subsequent release of pro-inflammatory mediators. Racehorses with naïve airways probably responded to lower endotoxin concentrations whereas
airways in horses that had adapted to cumulative prior exposures required much higher concentrations to induce a significant neutrophilic airway inflammation. Finally, although mould exposure is a major environmental factor implicated in a variety of respiratory disorders, \((1\rightarrow 3)\)-\(\beta\)-D-glucan, a fungal cell wall component, was not found to be associated with neutrophilic airway inflammation in this population of young racehorses.

In conclusion, results of this study indicate that endotoxin from dust sources (such as feed, bedding and sources outside boxes) in stables is associated with neutrophilic IAD in 2 and 3 year old thoroughbred racehorses in Australia. As in humans, the severity of this airway inflammation is related to the endotoxin concentration of the inhaled dust. Ventilation quality and meteorological conditions, particularly evaporation level, in looseboxes and stables also were significant risk factors for IAD. This has important practical implications, specifically for stable and ventilation design, and feed and bedding management. Horses housed in environments that encourage high dust exposure may be more prone to the early developmental stages of IAD and therefore as training progresses may be more likely to develop bacterial complications and clinical respiratory disease.
1. What is Currently Known About Inflammatory Airway Disease in Young Racehorses?

1.1 Introduction

In the last 10 to 15 years, there has been an enormous increase in interest in lower airway inflammatory disease in performance horses. Much information is now known about chronic inflammatory airway diseases, such as recurrent airway obstruction (RAO) 1, as well as infectious airway diseases caused by specific viruses and bacteria 2-4. However, little clinical or epidemiological research has been performed, particularly under Australian conditions, on acute non-specific lower airway inflammation, now termed “inflammatory airway disease”.

Inflammatory airway disease is a syndrome that broadly encompasses many infectious and non-infectious inflammatory disorders of the lower respiratory tract (LRT) 5,6. In many early cases, horses display few or no signs of respiratory tract dysfunction, such as a cough, whereas veterinarians performing routine LRT endoscopy often observe mucus in various quantities and with variable inflammatory cell infiltration in the trachea. However, in Australia, this acute syndrome has largely been unrecognised or underreported in young performance horses, which has led to a paucity of knowledge about exactly how common it is and what risk or causal factors are involved. Most important is that from a practical viewpoint even minor accumulations of mucus and inflammatory secretions could cause uneven distribution of airflow and impair gas exchange, particularly in racehorses during intense exercise 7, and this impairment of respiratory function is likely to be detrimental to peak performance.

There is little doubt that along with other respiratory diseases affecting performance horses IAD is one of the most important disorders facing the racing industry in this country, with immense potential for impact on performance, industry and horse owner/trainer economics, losses due to wastage, and welfare. With the majority of previous work concentrating on the association between infectious agents and IAD, 4,8-11, there is now a need to investigate the contribution of non-infectious dust agents, as well as stable management and environmental factors, to the induction of IAD in young thoroughbred racehorses.

In the following review of literature, the role of dust agents in the genesis of IAD in racehorses will be explored. The first part briefly outlines the structure and function of the lower respiratory tract with emphasis on cellular composition and the samples that are used to retrieve these cells for diagnosis of airway inflammation. The second part examines how and where dust agents are deposited within the lower respiratory tract and what mechanisms defend against inhaled noxious dusts. The inflammatory and immune cells involved in defence and their possible contribution to inflammatory airway processes also will be discussed. In addition, the specific and sometimes unique conditions racehorses are exposed to, which may result in inhalation of larger burdens of dusts and gases, imbalance defensive mechanisms and promote airway inflammation, will be reviewed. The final section will deal with the types of infectious and non-infectious agents associated with airway inflammation in horses highlighting the effects of inhaled dust endotoxin and (1→3)-β-D-glucan.
1.2 Structure and Function

The equine respiratory system is complex, intimately combining structural and physiological mechanisms to facilitate the acquisition of oxygen from the atmosphere, the elimination of carbon dioxide and other bioactive substances from blood\textsuperscript{12,13}, while efficiently disposing of agents of disease. The adult respiratory tract is a series of branching tubes with an overall surface area of 2000m\textsuperscript{2}\textsuperscript{14,15} in which horses exchange approximately 110,000 L/day of air at rest, and up to 1800 L/minute (expired minute ventilation) during intense exercise\textsuperscript{16,17}. The respiratory tract therefore provides the largest interface between the external and internal environments and for this reason is constantly exposed to high levels of potentially deleterious air contaminants such as inorganic and organic dusts, gases and other pollutants\textsuperscript{14,18,19}. Although an elaborate multistage defence system has evolved to deal with these potentially damaging extraneous substances, domestication of the horse, intensive stabling establishments for racehorses often in urban environments, and other racehorse management practices, has lead to respiratory challenges from unfamiliar substances and especially from increased quantities of noxious particles\textsuperscript{14,20}.

The equine respiratory tract has 2 major functional divisions\textsuperscript{12}:
1. A gas transport system comprising the nasal cavity, pharynx, larynx, trachea, bronchi and bronchioles. This system also warms, humidifies and filters inhaled air.
2. A gas exchange system comprising alveolar ducts and alveoli, maximised by a large surface area and a thin gas-exchange barrier.

Anatomically, the nares, nasal passages, pharynx and larynx make up the structures of the upper respiratory tract (URT), whereas the structures distal to the larynx, including the tracheobronchial tree (trachea, bronchi and respiratory bronchioles)\textsuperscript{15,20} and alveolar ducts and alveoli comprise the LRT.

1.2.1 Cell Composition of Airways and Peripheral Lung

The normal cellular structure and function of surfaces of the upper and lower respiratory tract is described in Table 1.1. The epithelial cells lining airways primarily provide protection against potentially harmful effects of respired debris. These cells are firmly affixed together by tight junctions that serve as a diffusion barrier and limit movement of molecules and ions between the airway lumen and submucosa\textsuperscript{21,22}. In the alveolar region, the tight junctions between epithelial cells separate to allow passage of lymphocytes, macrophages and polymorphonuclear cells, essential for alveolar defence\textsuperscript{23}. Epithelial cells synthesise and secrete substances that protect the tissue and provide a specific fluid environment for normal mucociliary propulsion and for alveolar macrophage function\textsuperscript{22}. Although the structure of the epithelial lining is similar in most species, the ciliated epithelium of the horse is distinctive in that it extends as far as the terminal and occasionally respiratory bronchioles\textsuperscript{18}, conferring a greater ability to clear foreign particles via mucociliary transport.

1.2.1.1 Tracheal Aspirates and Bronchoalveolar Lavage Samples

The cells lining the tracheobronchial tree and gas exchange region, in addition to substances secreted from mucus and serous glands as well as serum transudates, form the principle components of tracheal aspirate (TA) and bronchoalveolar lavage (BAL) samples, which are used for evaluation of inflammatory disorders in the LRT. The adequacy of these samples is confirmed by the presence of most of the cellular constituents of the relevant airway (see Table 1.1) from which the sample is taken. For example, the presence of macrophages and ciliated epithelial cells is a pre-requisite for interpretation of TA cytology whereas to ensure that recovery of pulmonary epithelial lining fluid is adequate, BAL samples should contain foamy surfactant and have excellent alveolar macrophage and lymphocyte cellularity\textsuperscript{24,25}.
The **cell composition of TA samples** is influenced by a number of physiological factors, which ultimately determines the diagnostic usefulness and interpretation of this sample. For example, TA cytology not only reveals cell accumulations due to localised tracheobronchial disorders but probably also represents many regions of the LRT due to rapid mucociliary movement of cells and secretions from all parts of the lung to the trachea \(^{26,27}\). **Different airway regions, particularly if inflamed, therefore may contribute diverse numbers of cells, or specific cells, and secretions in a non-homogeneous manner to the TA total cell pool** \(^{28,29}\). This is highlighted by TA samples obtained from horses with pneumonia or pleuropneumonia containing cells representative of these distal LRT diseases \(^{30}\). In addition, exercise, which commonly precedes TA collection in racehorses, may influence cell constituents and their proportions.
Table 1.1: Normal cellular structure and function of surfaces of the upper and lower respiratory tract. [Adapted from references Pirie, 1990 #80; Widdicombe, 2002 #90; Pirie, 1990 #6; Robinson, 1997a #4; Nowell, 1981 #81; Mariassay, 1992 #82; Bohning, 1992 #80; Lakritz, 1997 #82; Ainsworth, 1998 #87]

<table>
<thead>
<tr>
<th>Structure</th>
<th>Cell types present</th>
<th>Function</th>
<th>Response to excess noxious stimuli</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upper Respiratory Tract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudal nasal passages, pharynx, larynx, guttural pouches and sinuses</td>
<td>Pseudostratified columnar epithelium, mostly ciliated, consisting of goblet cells and submucosal glands.</td>
<td>Mucus and fluid excretion for maintaining epithelial hydration and provision of protective factors; mucociliary transport of cellular and non-cellular debris.</td>
<td>Increased mucus production and viscosity; fluid transudation; cilia beat to move mucus and particulate matter toward nose *Clinical: nasal discharge.</td>
</tr>
<tr>
<td><strong>Lower Respiratory Tract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trachea and bronchi</td>
<td>Pseudostratified columnar epithelium, consisting mainly of ciliated and small volume goblet cells and submucosal glands; small proportion of non-ciliated (Clara) cells, basal, serous and neuroepithelial cells and cells with microvilli; many macrophages; occasional neutrophil and lymphocyte.</td>
<td>Mucus (mostly from submucosal glands) and fluid excretion for maintaining epithelial hydration, provision of protective factors and mucociliary transport; fluid and electrolyte exchange; goblet cells serve as repositories of mucus, help maintain baseline level of secretion and respond only to local stimuli; basal cells anchor epithelium to basal lamina; neuroendocrine responses to hypoxia and noxious stimuli; immune surveillance</td>
<td>Neutralisation and removal of infectious and non-infectious particles; increased mucus production and viscosity; fluid transudation; increased neutrophil % *Clinical: nasal discharge, cough, decreased work performance.</td>
</tr>
<tr>
<td>Small bronchus</td>
<td>As for trachea and bronchi but fewer ciliated cells and no microvillus or basal cells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchiole</td>
<td>Decreasing ciliated column to cuboidal cells and increasing proportions (55-75%) of non-ciliated (Clara) cells; no goblet cells; lymphocytes in BALT; occasional neutrophil, many macrophages</td>
<td>As for trachea an bronchi</td>
<td>As for trachea an bronchi</td>
</tr>
<tr>
<td>Terminal bronchiole</td>
<td>Mainly non-ciliated cuboidal (Clara) epithelial cells; few ciliated cells and no goblet cells; many macrophages and lymphocytes, occasional neutrophil</td>
<td>As for bronchiole</td>
<td>Detoxification of inhaled compounds or bioactive substances reaching airways via blood; xenobiotic metabolism; increased production of immunoglobulin (IgG, IgA), increased neutrophil % *Clinical: nasal discharge, cough, tachypnoea, increase lung sounds, decreased work performance.</td>
</tr>
<tr>
<td><strong>Gas Exchange Region</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory bronchioles</td>
<td>Type I and II (± Type III) alveolar epithelial cells (pneumocytes), capillary endothelial cells, many lymphocytes and macrophages, occasional mast cell and neutrophil</td>
<td>Comprises the air-blood barrier, provide surfactant, serous secretions, proteins, chemotaxins and immunoregulation; metabolize biologically active substances; mast cells involved in Type I hypersensitivity</td>
<td>Reduction in surface tension; phagocytic and opsonitic clearance of particles and microorganisms; xenobiotic metabolism; increased production of immunoglobulin (IgG, IgA, IgE), increased neutrophil % *Clinical: nasal discharge, cough, tachypnoea, increase lung sounds, decreased work performance.</td>
</tr>
</tbody>
</table>
Exercise can increase secretions into proximal airways in horses, and preliminary studies performed by the author indicate that neutrophil proportions also are significantly altered in TA following some form of exercise.

The methods for obtaining TA samples in the horse have recently been standardised and, along with issues regarding laboratory handling and cytological interpretation, are described in detail elsewhere.

1.3. Airway Defences and Inflammation

Humans and other animals can survive only minutes without oxygen and as a result must continuously inhale air. The respiratory tract is therefore constantly exposed to foreign gases and particles along with the required oxygen within air and consequently multistage defence mechanisms are present to cope with these frequently hazardous substances. These mechanisms are interdependent and coordinated for optimal lung defence and under normal circumstances maintain the health and sterility of the LRT. Moreover, if failure of a single mechanism occurs, while risk of disease is increased, alternate defences to a certain extent still are able to protect the LRT. However, when interference with several defence mechanisms concomitantly occurs, or when the invading material (eg., dust, bacteria or bacterial endotoxin) is sufficiently pathogenic and in sufficient concentration, or if the foreign substance is unfamiliar to the host’s defences, inflammation and disease invariably results. There are three major lines of defence successively (but also concurrently) encountered by particles that enter the airways.

1.3.1. Mechanical Filtering of Air

The first line of defence is the progressive mechanical filtering of inspired air through the convoluted air passages of the URT and the conductive air passages of the LRT. The efficiency of this defence varies with the dimensions and anatomy of the airway, the pattern of airflow as well as with the size, mass, velocity and physical characteristics, (such as density, diameter and shape), of the inhaled substances, particularly particulate material. Inhaled particles are deposited in the respiratory tract by five mechanisms: impaction, gravitational sedimentation, Brownian diffusion, interception and electrostatic deposition.

During normal breathing, more than 95% of inhaled particles ≥ 5µm (eg., small hay and straw fibres, fine wood shaving fibres, sand, pollens, plant spores, and larger bacteria, such as Streptococci) are filtered in the nasal passages, pharynx and tracheal bifurcation as a result of collision and impaction between high velocity particles within the airflow and changing airway anatomy. In the nasal passages, soluble noxious gases are concomitantly removed and neutralised via buffering by fluid and protein found in nasal mucus, and the air is humidified and warmed before entering the smaller lower airways. Once a particle impacts upon the moist nasal respiratory epithelium, it is trapped by mucus and removed by ciliary transport. It is important to note that even though few particles larger than 5 to 15 µm enter the trachea and more distal airways, those travelling with high velocity and volume of air can reach these airways and induce an inflammatory response. This response may be cleared or worsen depending on the agents’ toxicity and concentration, particularly if particles contain or have adherent toxins such as bacterial endotoxin. In humans, increased airflow velocity and volume leads to greater deposition of particles of this size in the trachea and bronchi rather than smaller lower airways and this likely is the same in horses.
As air reaches the small bronchi and terminal bronchioles of the LRT, total airway cross-section increases and air velocity drops. Deposition of smaller particles between 0.5 to 5 µm (e.g., large viruses, fungal spores and small to medium sized bacteria, fine feed or bedding particle, smoke and motorised pollution) subsequently occurs by sedimentation onto airway surfaces under the force of gravity where, under certain circumstances, they may induce pulmonary inflammation.

In general, slow, deep breathing (at rest) enhances sedimentation and leads to relatively uniform deposition of particles throughout the LRT. However, rapid breathing with increased airflow as occurs in horses during exercise increases impaction in the larger lower airways producing high local particle concentrations (10 times more) around the bifurcation carina of these airways compared to airway walls. This pattern and nature of deposition of potentially harmful particles may therefore be an important determinant of distribution of inflammatory responses in the LRT of racehorses (e.g., diffuse or localised to airways with major branches) and likely will have some influence on the results of fluid samples particularly those obtained from larger airway bifurcations (e.g., tracheal aspirates from the tracheal carina).

When air reaches the level of the respiratory bronchioles and alveoli, most particles < 0.5 µm do not contact the respiratory epithelium and are expelled in exhaled air. However, because air velocity is very low in the gas exchanging structures, particles with a diameter < 0.1 µm (e.g., gas molecules, endotoxin molecules, viruses, proteins, combustion nuclei, ultra-fine particles) are subject to random thermal kinetic buffeting (Brownian motion/diffusion) and have time to diffuse to the walls of surrounding air surfaces.

The relative importance of the above three mechanisms for particle deposition in a given airway therefore depends primarily on particle size and density and velocity of air moving through the airway. However, turbulence induced by airway branching and surface irregularities, and the non-spherical characteristics of particles tend to blur the distinctions between the mechanisms. In addition, highly charged particles (such as those that are freshly generated by mechanical disintegration or are sprayed as liquid droplets) are deposited by electrostatic image forces induced on airway surfaces. As well, fibres often of similar dimensions to the airway, effectively resist impaction and sedimentation by aligning themselves with airstream lines and penetrate as far as the gas-exchange regions where turbulence causes the fibres to flip end over end and intercept with airway walls. This is an important deposition mechanism for such fibres as asbestos in humans but may also be important for specific fibres of feed origin (e.g., chaff slivers) present in horse’s stables. Other factors that can influence deposition of particles include hygroscopic swelling of droplets as a result of increased temperature and humidity within airways, and presence of pre-existing tracheobronchial disease, both of which can increase tracheobronchial deposition.

1.3.2. Epithelial Lining Fluids, Mucociliary and Alveolar Clearance

Once foreign substances are filtered and deposited at specific sites within the LRT a second line of defensive mechanisms trap and clear or neutralise the offending agent(s).

Epithelial lining fluids provide a physical barrier to gaseous and particle contact by lubricating, humidifying, waterproofing, insulating and providing an appropriate environment for normal ciliary clearance. The fluid lining also provides a selective barrier against certain macromolecules, entraps and binds micro-organisms and particles, provides an extracellular surface for immunoglobulin and enzyme activities, and neutralises toxic soluble gases.

The fluids that line the tracheobronchial airways are a mixture of tissue transudates and mucus and serous secretions discharged from the submucosal glands, goblet and other surface epithelial cells, which are interspersed with the ciliated cells of the surface epithelium. Once secreted, this “mucocolloid” separates into two layers coating the ciliated epithelium, a deep, low-viscosity, serous sol layer underlying a
viscoelastic gel layer in which long-chain glycoprotein mucins are entangled, resulting in the characteristic rheologic properties necessary for mucociliary clearance \(^{20,41}\). Each ciliated epithelial cell has approximately 200 cilia that beat more than 1000 times per minute in a coordinated wavelike pattern within the sol layer \(^{41}\). During an effective stroke, the clawed tips of the cilia extend and engage the overlying sticky gel layer and sweep mucus, along with any intermingled particles and other debris, proximally toward the pharynx. In recovery, the cilia bend and flex within the sol phase near the cell surface and do not propel the mucus \(^{15,18,41}\). In healthy humans and horses, mucus is continuously propelled to the pharynx at approximately 0.1 cm/minute in the small airways and approximately 2.0 cm/minute in the main bronchi and trachea \(^{20,27,41,42}\) where ultimately it is swallowed or cleared by coughing. On average, healthy humans can clear inhaled particles completely from the terminal bronchioles to the larynx in 6 to 8 hours \(^{20,43}\), representing the fast phase of airway clearance \(^{18}\). Clearance of the same structures in healthy horses probably takes about the same time although clearance from the small bronchioles may take only 0.5 - 1 hour \(^{44}\).

Tracheobronchial lining fluids contain approximately 95% water and approximately equal amounts of glycoproteins, proteoglycans, carbohydrate, lipid and organic material. The protein fraction mostly consists of mucoglycoprotein (mucins), albumin, immunoglobulin (Ig) A, IgG, IgM, IgE, lysozyme, other globulins and complement components \(^{20,45}\). These constituents are secreted actively and passively and localise in the sol or gel layer according to solubility where together they play a vital role in directly inhibiting, neutralising, opsonising and destroying bacteria, viruses and other pathogenic particles as well as recruiting inflammatory cells such as neutrophils, macrophages and lymphocytes. The terminal bronchioles and alveoli also are lined with fluids derived from types 1 and 2 pneumocytes, Clara cells and from plasma. These fluids contain many of the above constituents (apart from mucins), as well as the important surfactant lipids \(^{20,36,38}\). Surfactant prevents alveolar collapse by reducing surface tension but also can act as an opsonin and enhance particle phagocytosis and lysis \(^{38}\), provide direct antibacterial, anti-fungal and anti-viral action \(^{46}\) and suppress proliferation of immune cell functions \(^{47}\).

Only a very small proportion of extremely small foreign substances reach the alveoli and these are subject to slow alveolar clearance (half-time of days to hundreds of days) \(^{18,20}\). Deposited particles can move randomly out of the alveoli and onto the terminal portion of the mucociliary escalator or can bind to (eg., bacteria and bacterial endotoxin) or penetrate (eg., viruses) epithelial cells and be cleared similarly after shedding or death of the cell \(^{36}\). Alternatively, particles may infiltrate the interstitium and be removed by lymphatic drainage \(^{23}\). However, the most important alveolar defence mechanism is phagocytosis of particles by macrophages.

Although macrophages are especially numerous within the terminal bronchioles and alveoli, they are present at all levels of the respiratory tract and are the most common cell recovered from TA (mean relative %: 71-85) \(^{48,49}\) and BAL (mean relative %: 48-56) \(^{25,50-52}\) fluid samples in healthy horses \(^{14}\). In normal equine lungs, these mobile cells are adapted to an oxygen-rich environment \(^{53}\) and are the primary or sole phagocyte responsible for clearance of inhaled particles and microorganisms from the distal airways and alveoli. They efficiently remove and degrade these harmful particles and normally do not attract or activate other immune cells, thereby promoting inflammation \(^{14}\). However, in situations where other cells (eg., T- and B-lymphocytes) have been recruited and their inflammatory mediators secreted, macrophages are accompanied by increased proportions of neutrophils and/or other inflammatory cells.

Airway or alveolar macrophages reach sites of particle deposition by chance or through attraction to substances released by particles, activated lymphocytes, neutrophils and other macrophages, or to immunoglobulin or complement coating particles in alveolar fluid \(^{20}\). Macrophages engulf foreign particles or pathogens and digest or detoxify them by lysosomal enzymes or oxidation via generation of oxygen free radicals \(^{54}\). Macrophages also present antigenic material to T- and B-lymphocytes or plasma cells, thereby triggering cell-mediated and humoral defence mechanisms \(^{55}\). Alveolar macrophages are
removed from terminal airways by unknown mechanisms that allow them to reach the mucociliary escalator or by migrating through the interstitium into lymphatics or blood vessels.

It is important to note that because alveolar clearance generally is slow, indigestible particles can be sequestered in macrophages and released when macrophages die. This may allow certain noxious particles, pathogens or toxins to persist in lung, causing persistence and chronicity of airway inflammation, which may result in infection, immunogenic reactions or toxicity.

Two final defensive mechanisms allowing clearance of offending agents include reflex bronchoconstriction and cough. In response to chemical or mechanical irritation by pollutant gases or particles, sensory receptors in the airway mucosa, as well as local release of neuropeptides, cause reflex bronchoconstriction. Increased vascular permeability, increased fluid and mucus secretion, and chemotaxis of neutrophils and inflammatory mediators also occur. In addition, when mechanically deformed by inhaled dusts or accumulated cellular debris, receptors unevenly distributed in the mucosa of the tracheal carina and large bronchi induces cough, resulting in expulsion of material. Clearance of dust material by coughing is most effective in proximal lower airways (down to level of fourth or fifth generation bronchi). Coughing is especially important when inflammation is established (and irritant receptors are more exposed and sensitive), and when mucociliary transport is defective resulting in accumulation of mucus. However, it is noteworthy that in horses with airway inflammation and concomitant bacterial infection as a result of confinement and prolonged head elevation, coughing alone is insufficient to expel mucus from airways and postural drainage also is essential in preventing accumulation or airway debris.

1.3.3. Immunity and Inflammation

The third line of defence of the LRT involves multiple and complex interactions between particles and epithelial cells, lymphocytes (eg., T- and B-cells/plasma cells), immunoglobulins, phagocytic cells (eg., neutrophils, macrophages), other inflammatory cells (eg., mast cells and eosinophils) and also with the many proinflammatory mediators (eg., peptides, interleukins and cytokines) secreted by these cells.

Epithelial cells lining the LRT including the gas exchange region serve an important immune function upon contact with noxious inhaled particles that have penetrated the surface barriers described previously. They produce antimicrobial molecules such as defensins, recruit phagocytic cells by expressing surface adhesion molecules and secrete proinflammatory mediators. They also play a role in up-regulating and down-regulating the inflammatory response of activated neutrophils.

Inflammatory cells and mediators interact with all the physical defensive mechanisms discussed previously, chiefly via bronchial blood and strategic localisation of lymphoid aggregates in the pharynx and at sites of airway bifurcation. They are components of bronchial and alveolar fluid lining and can act solely, particularly at the alveolar/interstitial level, when particles uncommonly breach these structures' defences. The immune and inflammatory cells and their products and interactions have been thoroughly reviewed elsewhere and only issues emphasising immune and inflammatory responses to particles involved in airway inflammation and IAD will be discussed further.

1.3.3.1. Role of Lymphocytes

When organic particles and microorganisms deposited in the LRT penetrate the fluid barriers or breach the alveolar and interstitial defences, they gain access to lymphoid tissue (such as bronchial associated lymphoid tissue [BALT] or interstitial lymphoid nodules) and subsequently elicit systemic and local T-lymphocyte and B-lymphocyte (or antibody-mediated) immune responses. Lymphocytes are the second most common cell present in LRT secretions of normal horses (demonstrated by their recovery from BAL and TA samples). As a result of difficulty in accurately differentiating them
from small macrophages and epithelial cells in tracheobronchial secretions, they usually are found in lower proportions in TA samples (mean relative % in TA: 4-11%; in BAL: 31-40%) 

**T-lymphocytes** are the predominant lymphocyte in the equine [Rush-Moore, 1995 #114] and human lung, present within the airway lumen or localised to epithelium or specialised lymphoid tissue. They exist as T helper (Th) cells (Th-1 and Th-2), T cytotoxic (Tc) and T-suppressor (Ts) cells. Stimulated T-lymphocytes also differentiate into Natural Killer (NK) cells and memory cells.

**B-lymphocytes** differentiate into plasma cells, which produce all classes of immunoglobulin except IgM. Secretory IgA is the predominant immunoglobulin within proximal airway secretions and is crucial for local humoral defence particularly against adherence of extracellular bacteria (eg., Streptococci) to respiratory epithelium. In the distal airways, however, IgG concentrations are highest and along with IgA, IgM and IgE are produced by lymphoid tissues in airway walls and BALT or reach airways from serum. Immunoglobulin G binds to organisms, toxins and particles preventing epithelial attachment, acts as an opsonin to promote uptake and destruction of inhaled foreign particles, and activates complement, which destroys extracellular organisms and recruits and activates neutrophils, mast cells, basophils and monocytes.

**T-lymphocyte-mediated responses** are central to a variety of human airway disease such as asthma and chronic bronchitis and although incompletely studied in horses these responses likely are highly important in many “dust-induced” respiratory diseases (eg., neutrophilic/large cell IAD, heaves, and pneumonia) in this species as well [McGorum, 1993 #115; Rush-Moore, 1995 #114]. For example, inflammatory diseases characterised by high proportions in airways of eosinophils and/or mast cells, such as IAD, parasitic, or “allergy-based” inflammation in horses, as well as asthma in humans, suggests a Th-2 response. This response involves Th-2 secretion of specific cytokines, which enhances production of IgE and IgA and results in mast cell and eosinophil recruitment into airways [Tizard, 1999 #112].

In contrast, large numbers of activated macrophages and neutrophils in airways suggests contribution of inflammatory Th-1 cells and B-lymphocytes producing IgG. T helper-1 cells also secrete chemical mediators that result in recruitment and activation of macrophages, which in turn release mediators that attract neutrophils to sites of inflammation. This response may occur in neutrophilic inflammatory disorders such as IAD as well as in summer pasture-associated RAO in combination with a Th-2 response.

Other T-lymphocyte-mediated responses include Tc cell-mediated mechanisms for immunity to viral particles such equine herpes virus-1 (EHV-1), whereas other viruses and intracellular bacteria such as *Rhodococcus equi* (ie., Rattles) are recognised and killed by Tc and NK cells.

Although lymphocytes control cell-mediated and humoral immunity and macrophages are the primary phagocyte in the lung, recruitment of other inflammatory cells plays a major role in defence against pathogenic particles particularly when the number of particles exceeds the ability of the previous lines of defence to clear the particle burden. A complex network of cytokines released from airway epithelial cells, macrophages and T-lymphocytes, as well as the complement pathway rapidly attracts neutrophils, eosinophils, basophils and mast cells from pulmonary capillaries, airway epithelium and bronchial blood to sites of particle invasion. However, in some situations, influx of these cells and active inflammatory responses induced by them is uncontrolled or inappropriately regulated. Excessive release of toxic mediators by neutrophils and macrophages in particular may damage lung structures and contribute to clinical signs in several pulmonary diseases in animals and humans.
1.3.3.2. Role of Mast cells/Basophils

Mast cells are located predominantly in connective and submucosal tissue surrounding airways, mucus glands and blood vessels in normal equine \(^{14,38}\) and human lung \(^{38}\). Few are found in airway and alveolar lumina \(^{14,38}\). In horses, this is reflected by the very low proportions of these cells found in samples collected from proximal and distal lower airway sites (mean relative %: < 1% in TA and < 2% in BAL) \(^{33,49,52}\). However, these proportions may be higher (up to 7-9%) in distal lower airways \(^{25,51}\). Basophils are the circulating form of mast cells and have similar immune functions in airway submucosa as mast cells \(^{38}\).

When mast cells sensitised with IgE are exposed to specific inhaled particle antigens, degranulation of their granules occurs and preformed mediators such as histamine, serotonin, proteases, heparin and neutrophil and eosinophil chemo-attractants are released. Alternately, mast cells can release granule mediators as a result of histamine releasing factors derived from leukocytes, complement factors and hot and cold stimuli \(^{84}\). Mast cells also can produce arachidonic acid-derived mediators such as prostaglandins, thromboxane, PAF and leukotrienes. The combined effect is to initiate immediate Type I hypersensitivity inflammation with induction of smooth muscle constriction of airways, increased blood vessel permeability and plasma transudation followed by inflammatory cell influx, all of which aid to expel or neutralise inhaled particles \(^{12}\).

1.3.3.3. Role of Eosinophils

Eosinophils rarely are observed in healthy equine airways, accounting for < 1-2% of inflammatory cells in TA and BAL samples \(^{25,33,48-52}\). In the lung, they are mostly located in connective tissues \(^{85}\) and are attracted there and into airways by mediators released from mast cells, macrophages and Th-2 lymphocytes and by parasites and fungal antigens \(^{86}\). In humans and other species, eosinophils play an important role in hypersensitivity or allergic reactions to specific inhaled particle antigens (eg., asthma), and this likely occurs in horses as well \(^{87,89}\). Eosinophilic recruitment may be involved in late phase “allergic” airway responses following exposure to inhaled particle challenge in horses \(^{90,91}\).

In most species, eosinophils interact with antibodies and stimulated mast cells and when activated release cytotoxic and other substances to combat parasitic airway infections and to modulate mast cell-dependent reactions \(^{85}\). Eosinophil numbers frequently are elevated in TA and BAL samples collected from horses with lungworm (Dictyocaulus arnfieldi) or migrating roundworm (Parascaris equorum) infestation \(^{14,76,88}\).

1.3.3.4. Role Of Neutrophils

Neutrophils are sparsely numbered in healthy airways in most species and play only a minor role in primary responses to inhaled particles in normal horses \(^{14}\). They account for < 5% of inflammatory cells in distal lower airways (as measured by BAL samples) \(^{4,9,33,92}\). However, the respiratory tract is a neutrophil-rich organ because neutrophils are delivered in profuse numbers by bronchial circulation and reside for variable time as “marginated” cells or slowly moving through pulmonary capillaries \(^{93,94}\). Under certain stimuli, such as when particle challenge overwhelms the primary phagocytic response of macrophages in magnitude or virulence, or if endotoxin is inhaled, neutrophil adherence increases and many are chemo-attracted through the endothelium into the interstitium \(^{93,95}\). Subsequently, they migrate into traheal, bronchial and alveolar airways where they may quickly outnumber macrophages in secretions in these airways. In general, a number of chemotactic substances released or stimulated by activated macrophages, T-lymphocytes, neutrophils and inhaled particles (particularly bacteria and viruses) control neutrophil migration and activation.
During airway inflammation, neutrophils phagocytose particles, particularly if they are opsinised by complement or IgG. When activated, neutrophils release a variety of toxic proteases, elastases, and collagenase and oxygen radicals from intracellular granules, which kill ingested and adjacent particles and can neutralise endotoxin. At the end of their lifespan (about 3 days) and following resolution of particle-induced inflammation, dying neutrophils are ingested and removed from airways by macrophages. Complex cellular and humoral factors interact in the lung to ensure that this inflammatory response within the lung resolves completely and in horses will usually do so in the absence of overt clinical signs such as recurrent cough, pyrexia or signs of depression.

However, if neutrophilic inflammation persists, is inappropriate or is excessive and the ability to remove neutrophils by macrophages is compromised, release of toxic proteolytic enzymes occurs into airway tissues either locally or diffusely. This subsequently can result in detrimental effects on lung structures (particularly the extracellular matrix), degradation of immunoglobulins and cleavage of epithelial cell-surface receptors responsible for regulating inflammation. Furthermore, attenuation in airway defences and altered airway surface during neutrophilic inflammation may result in the up-regulation of surface molecules, which act as receptors for rhinovirus and a wide spectrum of bacteria respectively. Therefore, inflamed airways may be more susceptible to colonisation and infection than normal airways. In horses, this may explain an important aspect of progression of airway inflammation from a subclinical particle- and/or endotoxin-induced macrophage and neutrophilic response that under certain circumstances results in significant bacterial colonisation of airways and clinical signs of infectious IAD.

1.3.4. Factors Promoting Airway Inflammation

Healthy animals are repeatedly challenged by burdens of inhaled particles and successful clearance of these particles requires that airway defences, particularly inflammatory mechanisms, be precisely balanced to ensure an adequate response to the invading particle while simultaneously employing measures to limit the duration and intensity of the response. Disruption of this balance can occur if animals are exposed to highly virulent or toxic particles, if the magnitude of the inhaled burden is great, or if defences are compromised in some way. In horses, malfunction or imbalance of LRT defences is the most common factor predisposing to neutrophilic airway inflammation and its progression, although young racehorses in particular must cope with unique management and environmental conditions that may increase their exposure to potentially toxic particles. However, it is important to note that these factors can induce airway inflammation initially without there being concomitant clinical (or pathological) signs of respiratory disease, a common finding in horses. Other factors or persistence of the same factor must subsequently be present for inflammation to progress to a point in which overt clinical signs (eg., poor performance, persistent cough, purulent nasal discharge, systemic signs) and chronic manifestations (eg., infection, significant changes to quality and quantity of airway mucus, structural airway changes) of disease are apparent.

In racehorses, the risk of imbalance of LRT defences and subsequent development of neutrophilic airway inflammation can increase for several reasons. For example, racehorses are placed under repeated “stresses” such as transport, sales, variably intense training/racing regimens, intermingling and housing in poorly ventilated and dusty stables. Although investigations have been equivocal, and effects are highly variable between individuals, one major outcome of these stressful events is an increase in corticosteroid concentrations in serum and airways. This results in decreased macrophage number, viability and phagocytic ability. In addition, mucociliary function can be impaired and quantity and quality of airway epithelial lining fluid can be altered allowing inhaled particles to remain for longer periods and in potentially higher concentrations within airways and facilitating chronic release of toxic substances.
Racehorses frequently are transported long distances to training establishments or racetracks and may not eat or drink adequately during this time. Dehydration may reduce the depth of the sol layer of the mucociliary lining fluid and impair ciliary stroke recovery and clearance of particles. As well, prolonged transportation can reduce concentrations of alveolar surfactant, which may impair defences in this region. In addition, prolonged head elevation during transport results in accumulation of neutrophils, mucus and significant numbers of bacteria in the lower trachea, emphasising the importance of postural or gravity drainage in augmenting mucociliary clearance. The presence of neutrophils, and in particular lysed neutrophil and bacterial DNA, can increase mucus viscosity, making it less transportable. Exposure to pollutant gases, ozone and smoke during transport and/or at urban racetracks or stables may enhance airway secretion, deepening the sol and gel layers and impairing ciliary stroke and mucociliary clearance. Under these circumstances, mucus accumulation may occlude airways and result in local hypoxia, which coupled with a stress-induced increase in endogenous corticosteroids, depresses airway macrophage number and function. 

During intense exercise while training or racing, racehorses dilate and straighten their airways and markedly increase their ventilation rate. Air filtration and conditioning of the URT therefore may be bypassed and more variably sized particles and colder and dryer air likely gain deep access to the distal lower airways and alveoli. Training or racing on dirt tracks in particular can increase inhalation of dust. As well, aspiration of oropharyngeal secretions including resident aerobic and anaerobic bacteria significantly increases with single or repeated bouts of exercise, placing a greater burden on LRT defences. In addition, training of racehorses very early in the morning (4.00 am to 8.00 am; as occurs in the Sydney metropolitan region, Australia) may increase the volume of unconditioned, colder air entering the distal lower airways. Inhalation of colder and drier air into distal airways that are unused to exposure to these conditions results in bronchoconstriction, decreased mucociliary clearance of surface secretions and neutrophilic inflammation in humans, dogs and probably horses.

Strenuous exercise increases corticosteroid concentrations in serum for up to 24 hours, which impairs macrophage function and alters peripheral lymphocyte function. Exercise-induced pulmonary haemorrhage (EIPH), a common and repetitive problem in racehorses in work, also can initiate neutrophilic inflammation within distal lower airways. Finally, although humans may benefit from accelerated mucus transport due to increased airflow during exercise, a similar effect in healthy horses has not been demonstrated. However, there is evidence that proximal lower airways are more likely to contain increased airway secretions after exercise in poorly performing racehorses, suggesting that exercise in this species may play some role in mechanically increasing progression of secretions toward the larynx.

Several management and environmental factors also increase exposure of horses to toxic gases and particles or decrease defences, and therefore predispose them to neutrophilic inflammation. For example, because racehorses are housed for long periods sometimes on dry dusty bedding (eg., poor quality straw) and often in poorly ventilated looseboxes at racetrack stables, they are exposed to very high levels of dust, endotoxin and ammonia that would be unacceptable by human occupational health and safety standards. Variable temperatures and humidity in the environment and in stables, which is known to affect mucociliary function, may compound this situation. In addition, racehorses have frequent contact with one another in paddocks, during race meetings, or at stables during training, and possibly coupled with poor hygiene practices amongst personnel handling them ensure horses are exposed to ubiquitous viral pathogens. Although inhalation of viruses such as EHV-1 and EHV-4 usually results in subclinical infection, focal destruction and exfoliation of ciliated epithelium invariably occurs. This impairs mucociliary clearance rates by approximately 50 (herpes virus) to 100 (influenza virus) percent for up to 4 weeks. Viruses additionally may decrease surfactant levels, stimulate increased airway secretions,
decrease number and function of macrophages and enhance bacterial attachment and colonisation. The combination of these factors subsequently leads to neutrophilic airway inflammation in many racehorses, and may increase sensitivity or hyper-responsiveness of airways to further burdens of particulate matter sometimes for weeks after recovery. Finally, as a result of immature immune responses and lack of tolerance to inhaled particles (particularly bacteria and viruses), racehorses ≤ 4 years generally are more prone to inflammatory airway diseases (Powell, 1974; Wood, 1999; Burrell, 1996; Chapman, 2000; Christley, 2001; Newton, 1999).

There is little doubt that many young racehorses are likely to be prone to development of airway inflammation given the conditions in which they are commonly managed. In most cases, inflammation is characterised acutely by a predominance of neutrophils and is subclinical. Perpetuation of “stresses” and/or continued exposure to dust agents may consequently result in a progressive inflammatory disease state in which different inflammatory cell populations may predominate and clinical and possibly pathological signs invariably are manifest.

1.4. Causes of Inflammation

It is likely that a variety of aetiological agents acting simultaneously or sequentially are responsible for equine airway inflammation and the various clinical manifestations encompassed by IAD. Although the causal role of agents and the temporal relationship between these agents has yet to be completely defined using well-designed epidemiological studies, both infectious and non-infectious agents probably have important roles in the development and/or maintenance of airway inflammation and clinical disease. In the following discussion, broad details of infectious agents will briefly be summarised. However, emphasis will be placed on non-infectious agents, particularly the role of dust-borne endotoxin and \((1 \rightarrow 3)\)-\(\beta\)-D-glucan in the genesis of airway inflammation and IAD in horses.

1.4.1. Infectious Agents

Infectious agents such as fungi, bacteria and viruses are ubiquitous in most environments in which animals and humans reside. The most important sources of these agents in equine environments are the normal flora of the horse, the loosebox or stable and feed and bedding. When infectious agents become aerosolised and are inhaled, lower airway inflammation and disease may be induced. However, this depends largely on airborne survival of the pathogen (which may only be seconds for certain bacteria and viruses), the capacity of the microbe to cause damage or cell dysfunction (directly or via toxins or virulence factors), and the stresses the horse being exposed is under. In addition, potentially pathogenic bacteria and Mycoplasma that are commensal in the URT can inadvertently be introduced or aspirated into the LRT and under specific circumstances can initiate inflammation and clinical disease. This latter situation probably is the most common reason for IAD with a bacterial component in horses. Reviews on specific bacterial, fungal and viral species that have been associated with IAD in horses have been detailed elsewhere.

The viruses most commonly associated with upper and lower airway inflammation in Australian horses include EHV type 4 and less commonly type 1, equine rhinitis (ER) A and B viruses, and equine adenovirus. These viruses are enzootic in racehorse populations, are involved in latent clinical infections and are likely to increase sensitivity or susceptibility (particularly in young horses) to bacteria, fungi and other inhaled particles. In addition, outbreaks of EHV in training yards have been associated with neutrophilic airway inflammation. Equine influenza virus, absent in Australia, is also an important cause of prolonged impairment in tracheal mucociliary clearance in horses resulting in airway inflammation, and in some instances secondary bacterial infection or persistent airway hyper-reactivity. However, except for equine influenza, current epidemiological evidence indicates that there is no direct association between seroconversion to these viruses and signs of respiratory disease.
(ie., cough) or airway inflammation in racehorses. Instead, an indirect or longitudinal relationship may exist between viral infection, other agents, and development of persistent airway inflammation and/or hyper-reactivity.

Over 50 species of fungi and actinomycetes have been identified in stable air, the highest challenge from stable materials and feed being thermophilic and thermotolerant mould species. However, the most important organisms, in terms of their role in chronic airway inflammation (RAO), are Faenia rectivirgula (Micropolyspora faeni), Thermoactinomyces vulgaris and Aspergillus fumigatus. Exposure to fungal spores is associated with allergic IgE-related and non-specific neutrophilic inflammation in lower airways of horses and humans, which may result directly from inhalation of fungi or from inhalation of volatile chemicals produced by fungi, such as alcohols, aldehydes and ketones. Inhalation of fungal cell wall components such as \((1\rightarrow3)-\beta-D-glucan\) may also be involved in modulation of airway inflammation (see later section).

In contrast to viral and fungal agents, bacteria and mycoplasma principally enter the LRT from inhalation of commensal flora in the nasopharynx. Although large concentrations of airborne bacteria (eg., up to \(1.9 \times 10^4\) bacterial colony forming units (cfu)/m\(^3\) Gram negative organisms) may be found in stable environments, most are likely to be non-pathogens and on average only \(\sim 10\%\) are viable. Strong association exists between cultures of significant numbers of specific organisms in lower airways and neutrophilic airway inflammation in racehorses, as determined by TA. The specific bacteria, associated with airway inflammation include Streptococcus zooepidemicus, S. pneumoniae, and Actinobacillus/Pasteurella spp. Similarly, a significant and strong relationship is present between isolation of >10\(^3\) cfu/mL from TAs, of Streptococcus zooepidemicus, Streptococcus pneumoniae, Streptococcus suis, Streptococcus sanguis, Actinobacillus/Pasteurella spp. and Bordetella bronchiseptica, and coughing in young racehorses, coughing being a specific indicator of neutrophilic lower airway inflammation. However, it is important to realise that these bacteria have been found only in 65\% and 42\% of horses with airway inflammation or coughing respectively, the remaining horses developing an airway inflammation probably of non-infectious aetiology.

Several Mycoplasma species (M. felis and M. equirhinis) also are associated with airway inflammation in horses in training in the UK, although this relationship is not found in young racehorses in Australia. In contrast, Staphlococcus spp, Corynebacterium spp and anaerobes, also found as normal flora of the URT, are not associated with acute lower airway inflammation or coughing.

A temporal relationship has not been determined between LRT bacterial infection and onset of airway inflammation, and it is argued that presence of bacteria may reflect impaired mucociliary clearance of airways for some other reason. However, experimental inoculation with Streptococcus zooepidemicus and Streptococcus pneumoniae into the trachea of horses causes airway disease indistinguishable from IAD. In addition, when the criteria for causality outlined by Bradford Hill (including strength of association, specificity, consistency, biological gradient and plausibility, coherence, experimental evidence and analogy with other species) is applied to well designed epidemiological studies investigating the association between bacteria and airway inflammation and coughing most categories are satisfied. These issues therefore lend strong support to the hypothesis that bacteria are important aetiological infectious agents in IAD in young racehorses.
1.4.2. Non-infectious Agents

The association between exposure to foreign particle and gaseous substances within air and respiratory disease in humans has been documented for hundreds of years [Magnus, 1555 #195; Floyer, 1726 #196; cited by Rylander, 1994 #194], and more recently has been demonstrated with epidemiological studies in many industrial and agricultural environments 155-157. Intensive research over the last two decades within the production animal industry also has revealed a strong association between poor air quality and reduced production and increased inflammatory respiratory disorders 158-160. It is not surprising therefore, that exposure to non-infectious dusts and gases in stables play a significant role in the genesis, severity and duration of airway inflammation in horses as well 7,126,161,162. Although levels of dusts in horse stables are lower than in intensive livestock buildings, and horses are housed in large individual boxes with greater labour per animal 57,163, horses probably are more sensitive to dust inhalation than other species 164 and exposure is measured in years rather than months 148. However, the relative contribution of environmental and management factors and specific non-infectious dust and gaseous agents over time (longitudinally) to development of airway inflammation in young racehorses housed in stables is unknown and is the major focus of this report.

Particulate agents are defined as any small particles or droplet, inorganic or organic, viable or non-viable, which can become airborne and be inhaled 43. They can range from small molecules less than 0.001 µm to pollens and spores ranging between 2 and 50 µm to very large visible particles in the range of 1000µm 43. Particles also assume many regular and irregular shapes, and while most occur naturally, many are the result of alterations in human activity 43. Finally, particles can be infectious or non-infectious, innocuous or directly toxic or highly allergenic, whereas others act as vectors carrying toxic substances such as bacterial endotoxin on their surfaces.

1.4.2.1. Sources and Constituents of Inhaled Particulate Matter

Humans and racehorses are constantly exposed to a wide range of dust agents, which broadly can be grouped into inorganic and organic components. The complex inorganic fraction is derived chiefly from soil silicates and quartz, both of which have been associated with inflammatory respiratory diseases in humans 155. Exposure to these agents is highly likely in horses worked on dry dirt tracks, stabled on sand bedding and in those that are exercised using mechanical walkers, the surface of which is usually covered in a layer of dirt or dust. In contrast, the organic dust fraction is airborne and settled material of biologic (vegetable, animal or microbial) origin. It contains a heterogeneous mix of plant matter, shed epithelial cells, mites, parts of insects, rodents and birds and their excreta as well as inorganic matter and dung dander 155. Bacteria and their biochemical components and excretions, (such as endotoxin, peptidoglycan and proteolytic enzymes), moulds, fungal cell wall components (such as (1→3)-β-D-glucan), fungal spores and mycotoxins, 148,155,165 also are major components of organic dust matter given suitable nutrient supply, pH, aeration, water and temperature 166.

In racehorse environments, particularly stables, organic dust agents are derived from the horse and its excretions (eg., dried faeces, skin, dander and hair) 148,167,168 as well as construction materials of the stable, and stable vicinity where human and vehicular activity contributes to generation of airborne dusts. However, type of feed (eg., hay, grains, pellets and silage) and the conditions in which it was harvested and stored, as well as the type of bedding on which horses are housed (eg., straw, coarse and fine pine woodchip shavings) are the most important determinants of the variety and quantity of dusts found in horse’s stables 7.

For example, the constituents of commercial feeds such as alfalfa cubes and pellets generally contain fewer mites and their faeces, fungi, actinomycetes and fungal spores (particularly those incriminated in inducing chronic airway inflammation or RAO) than hay and haylage 139,169. Hay that has heated during storage or when baled at 35-50% moisture content, may contain very high levels of bacteria, mould
spores, actinomycetes and forage mites and their pro-inflammatory products such as endotoxin, (1→3)-β-D-glucan, proteinases and mite faeces 127,170.

Visibly dusty hay generates 10 fold more respirable particles in looseboxes than good quality hay, which in turn yields approximately 10 times the respirable particles of processed silage and alfalfa pellets 171. As well, dry rolled grains of barley and oats liberate 2 to 30 to 60 times more respirable particles than hay, whole grains, or grains mixed with molasses respectively 171. In general, wet alfalfa timothy hay and commercial alfalfa cubes, haylage and pellets generate lower respirable dust concentrations in horse looseboxes than dry alfalfa timothy hay 169,172 whereas commercial alfalfa cubes produce lower total breathing zone dusts than hay 172. It is important to note that feeds that can potentially liberate high concentrations of dusts into the air likely will also contain higher concentrations of dust constituents (including pro-inflammatory agents and aeroallergens), and the challenge dose from these feeds will be even greater in the breathing zone of horses 127.

When straw is baled with high moisture content, or if used in deep litter systems in poorly ventilated stables, it may develop very high concentrations of bacteria, mould spores and endotoxin 18. Although commercial wood shaving, sawdust and paper bedding is generally promoted over straw as low-dust bedding 126,127,173, wood shavings and sawdust may generate higher respirable dust concentrations than fresh, clean straw and flax straw 19,171. However, straw bedding usually generates higher concentrations of total airborne bacterial endotoxin than phone book paper and wood shavings/sawdust bedding, although concentrations of airborne gram-negative bacteria produced by these bedding types are similar 174. It is important to also note that racehorses kept on straw bedding are twice as likely to develop lower airway inflammation than horses housed on shredded paper, and recover more slowly from this airway inflammation 8.

Horses housed on deep litter wood shavings bedding, or on bedding that is inconsistently changed and cleaned of excretions (as may occur in some poorly managed racetrack stables), may be exposed to higher concentrations of fungi and actinomycetes, which multiply in degrading wood shavings, (particularly pine wood) and stable dung 127,175,176. These agents are known to exacerbate airway inflammation in heaves-affected horses 162,177. Another factor contributing to high release of spores and probably other particles from bedding such as straw, wood shavings and paper is high humidity combined with high temperature 176,178, a common weather pattern found in metropolitan racetracks in Sydney, Australia. Finally, when comparing the relative contributions feed and bedding make to the overall airborne dust load in looseboxes, wood shavings and other bedding types generate significantly less respirable dusts than hay and dry, rolled grains of barley and oats 171.

1.4.2.2. Noxious Gases

Housed animals are exposed to a range of potentially harmful gases including ammonia and hydrogen sulphide, which act as irritants, and methane and carbon dioxide, which act as asphyxiants 179,180. Exposure to ozone, sulphur dioxide, carbon monoxide and nitrogen dioxide also occurs 157, particularly in urban environments in which automobile and industrial pollutants and emissions can be high. This is an important source of exposure to gaseous airway irritants in humans and may equally be important in racehorses residing in racetrack stables within cities, as occurs in Sydney, Australia.

It is important to note that much of the information regarding noxious gases comes from studies investigating acute and longitudinal exposures in intensively housed animals 181,182 and laboratory animals 183,184, and occupational or air pollution exposures in humans 157. Increased concentrations of noxious gases are associated with non-specific airway inflammation and impaired lung function in humans 157,185 and in intensively kept animals 182,186, and threshold limiting values (TLVs) for most of these gases have been determined 187,188. However, except perhaps for ozone 189-191 and ammonia 192, there are no scientific
studies demonstrating that exposure to noxious gases in stables or outdoor air pollution plays an equivalent role in equine airway inflammation, and TLVs for any noxious gas for horses are unknown.

From work in other species, some general comments may be made about exposure to noxious gases (above TLVs), which possibly are applicable to horses living in stables. Most noxious gas molecules, particularly ammonia, tend to be deposited in the upper and proximal lower airways where they primarily cause mucosal irritation, inflammation and dysfunction, promote mucus secretion and airway narrowing \(^{157}\), and disrupt defences against other inhaled particles \(^{19}\). In contrast, inhaled ozone and oxides of nitrogen can penetrate as far as the gas exchange regions and cause injury to type I epithelial cells and endothelial cells \(^{193}\). Long-term low-level exposure to noxious gases more commonly results in airway inflammation than acute exposures \(^{19}\), although the latter situation is rarely encountered in horse environments. Individual variation in biological response to gas exposure, found often in humans \(^{194}\), probably also occurs in horses. In addition, many noxious gases probably act additively or synergistically with other inhaled non-infectious or infectious agents \(^{185}\), or exacerbate pre-existing disease processes \(^{19}\) such as asthma \(^{195}\), or modulate T-lymphocyte responses and decrease pulmonary immunity \(^{16}\). Finally, inhalation of gases may promote allergic airway inflammation by increasing allergenicity of allergens \(^{197}\) and by priming eosinophils for subsequent activation by inhaled allergens \(^{198}\).

Only a few studies have investigated effects of ammonia and ozone in horses. Compared to humans and laboratory animals, horses are less susceptible to acute effects of ozone and, despite minor evidence for a role in lower airway inflammation \(^{189,191}\), ozone is unlikely to be a significant risk for development of IAD in normal racehorses \(^{19}\). In contrast, horses exposed to high concentrations of ammonia (up to 130 parts per million [ppm]) in looseboxes may develop upper and proximal lower airway mucosal inflammation and clinical signs proportional to the concentration of inhaled ammonia \(^{192}\). Concentrations of ammonia rise in stables when deep litter bedding is used \(^{199}\); if looseboxes are poorly ventilated \(^{200}\) and where there is poor drainage of urine \(^{37}\), conditions that infrequently arise in racetrack stables in Sydney but may be more common in colder climates where ventilation often is limited. In addition, concentrations of ammonia may vary widely as a result of measurement technique (by a factor of 10), between looseboxes and areas within looseboxes, and may rely more on stable ventilation and horse activity than removal of urine and manure contaminated bedding \(^{194,200}\). Furthermore, ammonia concentrations vary depending on height above bedding with concentrations approximately two-fold higher at floor level (25 to 50 ppm 10cm above bedding versus 15 to 35 ppm at 105 cm above bedding), increasing exposure in recumbent horses \(^{199}\). Concentrations as high as 740 ppm have been recorded in poorly ventilated regularly mucked out looseboxes \(^{200}\). Finally, concentrations of ammonia at the level of the horse’s halter (breathing zone), in contrast to dust concentrations, are less or much the same as airborne concentrations in the loosebox at head height (105 cm above bedding) \(^{199}\).

Ammonia may enhance the inflammatory response in airways induced by other dust agents, particularly endotoxin \(^{201}\) and bacteria \(^{202,203}\), and therefore may be an important additive component in the generation of IAD in racehorses. The TLV for ammonia in humans for 8-hour/5-day week is 20 ppm \(^{187,204}\), with a short-term (10 to 15 minutes) exposure or ceiling limit of 35 ppm \(^{155,188}\). Recommended maximum levels for pig sheds is < 7 ppm \(^{158}\). Another noxious gas present in animal houses including horse stables is carbon dioxide. However, concentrations generally are assessed as an indicator of ventilation rate and of the general contamination of air inside livestock buildings \(^{205,206}\) rather than as a determinant of risk of respiratory disease. Although the TLV for carbon dioxide is recommended to be 1540 ppm, above which symptoms of respiratory disorders occur in workers in swine buildings \(^{207}\), higher and lower concentrations have been suggested for other industries \(^{187}\). In addition, ventilation rates based on carbon dioxide concentrations are not correlated with levels of ammonia, dusts and endotoxin concentrations and therefore do not account for ventilatory differences resulting from manure and urine management, feed and animal management and
other factors that affect production of contaminants other than carbon dioxide. For this reason, use of carbon dioxide concentration in horse stables probably should only be used as a crude measure of ventilation rate.

1.4.3. Factors Contributing to Dust Exposure

In order to be inhaled and cause an effect, dusts must be aerosolised such that solid particles (dusts or smoke) or liquid droplets (mists) of sufficiently small diameter maintain stability as a suspension in air. In racehorse environments this is achieved by machinery (e.g., mechanical walkers), ventilation air, or movement of humans and horses in and around stables. Particles that are aerosolised (e.g., large viruses, fungal spores and small to medium sized bacteria, fine feed or bedding dust, smoke and motorised pollution) usually range between 0.1 to 10 µm in diameter. They are cleared from air via direct removal by ventilatory airflow (particularly small fungal spores), removal of pathogenic potential while airborne (e.g., death of infectious agent) or by reaching an equilibrium and subsequently falling out of the air. Although aerosolised bacteria and viruses may die within seconds, viruses being particularly sensitive to changes in relative humidity, many species still maintain their pathogenicity, antigenicity and ability to induce airway inflammation. This is an important mechanism for certain bacteria, which after death release endotoxin and (1→3)-β-D-glucan, potent pro-inflammatory agents when inhaled into airways. In general, clearance of airborne dusts takes many hours whereas gases such as ammonia or ozone diffuse through air and can remain airborne in still air for much longer.

Racehorses, who spend most of their time (up to 22 hours) in looseboxes, are therefore exposed to aerosolised foreign dusts and gases almost continuously and any effects these agents have would likely be cumulative. However, it is important to note that these “steady state” dust levels do not reflect the true levels to which racehorses are exposed due to large variation induced by horse behaviour in looseboxes, horse and human activity in and outside looseboxes, and stable ventilation, bedding type and management. This large variation in concentrations of dusts can occur within horse looseboxes, between looseboxes in the same stable and particularly around the horse’s head.

For example, dust concentrations in the breathing zone (i.e., a hemisphere of 300 mm radius extending around the nostrils) of horses housed in conventional looseboxes (i.e., hay feed, straw bedding with adequate ventilation) can be 7 to 21 times higher respectively than basal loosebox concentrations as a result of considerable amounts of time eating with their muzzles in close contact with feed and sometimes bedding. Airborne concentrations of dusts in looseboxes, (mostly small fungal and actinomycete spores), regardless of quality of ventilation, can be 2-50 times higher when bedding in looseboxes is being changed and cleaned. On average, this increase in concentration is higher when horses are bedded on straw rather than wood shavings or paper. As well, short periods lying and resting on dust-rich bedding exposes horses to massive quantities of infectious and non-infectious particles.

Sweeping laneways or stable corridors, catching and moving horses, delivery of feed and bedding to stables, and proximity to roads and urban environments also contribute to increasing exposure of horses to airborne dusts. In addition, airborne concentration of dusts is significantly higher during the day, due to increased stable activity, than at night. During the day, aerosolisation of larger dust particles is reduced and equilibration and settling of these airborne dusts takes place. Although this results in decreased concentration of large size dusts, concentrations of very small dust particles remain similar or greater than values during the day, which ensures that racehorses in looseboxes continue to be exposed to pathogenic dusts. Finally, although not well studied in horses, ambient temperature, relative humidity and wind may alter airborne dust concentrations with hot, dry and/or windy conditions increasing exposure to dusts. Low temperature and humidity (<60%) can significantly reduce viability of most microorganisms. Conversely, increased humidity promotes fungal and bacterial growth in bedding and on walls, with subsequent spore and endotoxin elaboration, and may also increase moisture content of airborne dusts and increase their settling rate. Additionally, high ambient
temperatures during summer can decrease pig and probably horse movement in boxes resulting in less dust aerosolisation and exposure 219.

The recommended dimensions and airspace volume for looseboxes per racehorse in England to maximise air exchange rates/hour and decrease airborne dust burdens are 3.6m $\times$ 3.6m and 50m$^3$ respectively 220. Although these values are comparable to dimensions found in most Australian racetrack stable boxes (Malikides pers comm, 2003), it is important to emphasise that despite adequate size other stable factors contribute to quantity and composition of dusts and gaseous substances and exposure of horses to these dusts and gases. Natural and artificial ventilation rates inside looseboxes and within stables aerosolise organic dusts and delay their settling, although improved stable airflow results in reduced overall dust and gas concentrations 178,211. However, in situations where dust concentrations become higher outside stables or looseboxes, which may occur during intense human and horse activity at specific times at racetrack stables, natural ventilation will not be sufficient to clear indoor airborne dusts and in fact may increase the burden of airborne dusts within looseboxes 211. Furthermore, most stables reliant on natural ventilation, stables with ventilation rates less than 4 air changes/hour, or stables in which doors and shutters must be closed are inadequate to control dust and gaseous air pollution 178,222,223. This may be an unfortunate consequence of climatic conditions, particularly in the Northern hemisphere where stables are closed during winter, resulting in higher concentrations of dusts and gases in looseboxes during winter rather than summer 211. In intensive animal houses, dusts tend to be held in suspension without being removed due to a lack of strong updraughts in buildings and exhaust of dusts through high level vents 158, a situation that would likely apply to many equine racetrack stables, particularly those in Australia.

Horses housed in stables in which ventilation is poor are therefore exposed to elevated concentrations of airborne dusts, and can subsequently develop increased tracheal mucopus 126 and lower airway inflammation, usually without clinical signs of respiratory disease 224. Workers in various industrial and agricultural environments, such as production animal farms, cotton and grain mills, also are exposed to high airborne particle concentrations and are prone to development of airway inflammation 135,225-228.

1.4.4. Pathogenicity of Non-infectious Agents

There are a number of factors that dust or gaseous agents, and the hosts that inhale them, possess that may determine their pathogenicity or capacity to cause airway inflammation and damage. For example, many dusts act as vectors, carrying adsorbed pathogenic substances, such as endotoxin, aeroallergens, gases or microorganisms, either in or on the particle surface, deep into the LRT to activate an inflammatory response 148. Inhaled dusts therefore exert adverse inflammatory effects within airways either directly, due to sufficiently high toxicity or duration of exposure to overcome defences (Haber’s Law) 19, or indirectly via these adsorbed components. Recent research in humans however, suggests that the effects of organic dust exposure are most likely to be mediated by biologically active, specific agents in or on inhaled dusts 208.

Deposition of inhaled dusts and their components within airways can stimulate several adverse inflammatory effects. Most commonly in horses, deposition of dusts triggers “non-specific” inflammation that is dominated initially by neutrophil infiltration into airways in response to activated macrophages that release chemotactic cytokines 94. However, airborne particles, such as a wide variety of pollens, pollen fragments, mould spores and mite components, may have allergic capabilities and stimulate a specific allergic or Type I hypersensitivity inflammation with Th-2 and IgE-mediated infiltration of eosinophils and mast cells. This type of airway inflammation is manifest as asthma in humans 43, probably as a form of IAD in young horses 75,77,89 and RAO in heaves-susceptible horses 229. Host genetic factors also are extremely important in determining whether an allergic IgE-mediated response will occur, particularly in relation to asthma in people 195 and chronic hypersensitivity bronchitis or RAO in heaves-susceptible horses 230.
Another important pathogenic factor is that some particle material and particularly proteins that serve as allergens, have certain characteristics (e.g., molecular complexity, high solubility and stability) and often specific abilities to breach mucosal defence mechanisms and gain access to subepithelial sites controlled by the immune system. Other substances such as ozone or sulphur dioxide gases are characterised by high reactivity and concentration, which can create a diffusion gradient flowing from the centre of the airway lumen to the wall of the airway, resulting in a direct toxic effect on airway epithelium.

Finally, particles may act synergistically or additively with other non-infectious or infectious agents to produce airway inflammation. For instance, exposure to a potential allergen together with or preceded by exposure to cigarette smoke, industrial pollutant, viral infection or endotoxin may markedly enhance its allergenic potential and response, possibly by increasing epithelial permeability and allergen access to the immune system. A similar mechanism may occur in horses with viral respiratory tract disease, which frequently exacerbates hyper-reactivity and bronchoconstriction in response to inhalation of otherwise innocuous concentrations of particles or aeroallergens. Alternately, inhalation of allergens increases airway biological responsiveness to endotoxin. In addition, moulds and their cell wall component, (1→3)-β-D-glucan, commonly present in organic dust matter, can act synergistically with endotoxin or other pro-inflammatory agents to initiate or prolong airway inflammation. Finally, exposure to concentrations of ammonia of up to 100 ppm can intensify the damaging effects of certain bacteria in pigs and such an effect might occur in horses as well.

The complex mixture of particle materials that make up organic dusts means that it is very difficult to identify not only the individual agents that may cause airway inflammation and dysfunction but also their specific biologic activities. However, while research on these individual agents and their biological mechanisms in humans is growing, only recently have investigations in horses illuminated some of the characteristics of specific agents associated with inhaled dusts, (e.g., bacterial endotoxin), which may induce airway inflammation and subsequently clinical and pathological disease.

### 1.4.5. Specific Agents

Knowledge of specific agents in organic dust matter and their inflammatory effects in airways is limited in humans, and particularly so in production animals and horses. Examples of known agents, which when inhaled can act as allergens or be directly inflammogenic within airways include endotoxins, (1→3)-β-D-glucan, animal-derived proteins, tannins from leaves and woody plants, protease enzymes and fungal mycotoxins. It is very likely that several agents are unknown. However, bacterial cell wall endotoxins and fungal wall (1→3)-β-D-glucan are universally present in many types of organic dust matter and over recent years have been widely researched and reviewed as environmental and occupational hazards to humans. The importance of these two specific agents in equine respiratory health and disease will be further discussed.

#### 1.4.5.1. Endotoxin

Endotoxin is present in the cell walls of gram-negative bacteria and some blue green algae, both of which are very common in nature. The cell walls of gram-negative bacteria can be divided into three distinct layers, an innermost cytoplasmic membrane, a middle layer composed of peptidoglycan, and an outer layer, which contains the unique constituent lipopolysaccharide (LPS). Lipopolysaccharide contains an innermost lipid component, lipid A, which is highly conserved among Gram-negative bacteria and gives LPS its potent pro-inflammatory biological activities. Each genus and family of gram-negative bacteria produces a unique LPS, only some of which have the capacity to trigger airway inflammation. This may have important implications with regard to which of the common gram-negative bacteria found in organic dusts in equine environments as well as those that exist as normal flora in the oral and nasopharynx of horses, can induce airway inflammation and to what degree. It is important to distinguish between “endotoxin” and “lipopolysaccharide”, endotoxin being the term used in this report. Endotoxin is the form found in nature and refers to fragments of gram-negative bacterial cell walls that...
contain LPS as well as all the other naturally occurring compounds in the cell wall \(^{247}\). Lipopolysaccharide refers to the chemically purified substance with no trace of cell wall proteins \(^{247}\) that is commonly used in inhalation studies and in assays measuring “endotoxin”.

Gram-negative bacteria and endotoxin are ubiquitous in nature and are commonly present on surfaces of plants and animals \(^{248}\). Variable concentrations of endotoxins are found in agricultural environments such as housing for pigs \(^{207}\), chickens \(^{249}\), cows \(^{226}\) and horses \(^{250}\), as well as sources such as bedding, hay, grains, straw \(^{225,251}\), and bird droppings \(^{252}\). Endotoxins also are present in industrial processing and storage of organic materials \(^{248}\) such as cotton and flax, wood chips, timber, fibreglass, wastes, potatoes and paper, as well as in animal slaughter and processing facilities, domestic water \(^{253}\), house dust contaminated by pets and vermin \(^{254}\), and cigarette smoke \(^{255}\). Most environments in which horses have contact potentially can be contaminated with dusts with adherent bacteria and their cell-wall fragments, which subsequently may become aerosolised and inhaled. In addition, endotoxins are present in the oral, pharyngeal and nasal cavities \(^{239,248}\) as well as the intestinal tracts of humans and animals. Aspiration of secretions from the mouth and nasopharynx may lead to bronchial contamination by endotoxins \(^{256}\) although horses are more likely to be exposed to endotoxin-contaminated dusts via inhalation.

1.4.5.1.1. Specific Mechanisms of Inflammation Induced by Endotoxin

There are now overwhelming lines of evidence indicating that endotoxin is a fundamental component of organic dust matter, which, when inhaled, contributes to acute and chronic airway inflammation and airflow obstruction in humans. Recent evidence indicates that inhaled endotoxin also plays a similar and vital role in development of acute airway inflammation in exposed horses housed in stables and probably in the development of RAO in heaves-susceptible horses \(^{128}\).

In general, ambient air contains very low concentrations of endotoxin (±0.4 ng/m\(^3\)) \(^{257}\), and under normal circumstances, the lung has efficient defence mechanisms against airborne endotoxin. However, when dusts containing endotoxin are inhaled and deposited within airways in high concentrations, non-specific inflammatory mechanisms ensue within airways and also systemically \(^{258}\). In airway lining fluid, Lipid A is bound by lipopolysaccharide binding protein (LBP), an acute phase protein produced by Type II epithelial cells and extravasated from the vascular compartment \(^{259}\). The LBP-bound endotoxin is transported and attached to specific receptors on airway macrophages for metabolism and destruction \(^{260,261}\). Subsequently, endotoxin-bound macrophages are activated \(^{262}\), endotoxin is internalised and production of a variety of inflammatory cytokines is initiated \(^{263}\).

The major result of these processes in laboratory animals \(^{264,265}\), humans \(^{266}\) and horses \(^{128}\) is a significant elevation in concentrations of inflammatory mediators in airways \(^{267}\) and subsequent influx of neutrophils first into lung tissue within hours of inhalation then into airways after 4 to 24 hours. As well, numbers of leukocytes, principally neutrophils, increase in blood, an effect that also occurs in horses exposed to endotoxin-containing hay/straw \(^{128}\). However, this effect probably is dependent on the concentration of inhaled endotoxin and time of measurement, higher concentrations resulting in decreased blood neutrophil numbers due to margination and pulmonary recruitment of leukocytes \(^{128,268}\). As a consequence of neutrophil infiltration of airways, and as a direct effect of inhaled endotoxin, secretion of increased quantities of mucosubstances by mucus goblet cells occurs \(^{269,270}\), the excess luminal mucus persisting within airways for several weeks even if continued exposure to inhaled endotoxin desists \(^{271}\). Further effects of activated neutrophils are described previously.

It should be noted that genetic differences in the above cellular mechanisms responsible for toxicity of endotoxin, might result in variability between individuals, including horses, in their response to inhaled organic dusts \(^{240}\). In addition, although similar acute mechanisms likely occur in equine airways in response to inhaled endotoxin, conclusive evidence in IAD is scant. Procoagulant activity and concentrations of albumin, IgA, IgG, but not prostaglandin E\(_2\), (PGE\(_2\)) or PGF\(_{1\alpha}\), are increased in horses.
with IAD compared to controls but none of these (or any other) metabolites or cytokines have yet been studied in association with concentrations of inhaled endotoxin.

Although some of the acute physiological responses to inhaled endotoxin in airborne dusts outlined above are known, chronic effects or persistence of neutrophilic airway inflammation is not well understood. Whereas inhalation studies show that neutrophil infiltration into airways is a transient process triggered by each LPS exposure, in life, long-term exposure to variable concentrations of endotoxin occurs, which may result in unpredictable chronic inflammatory responses. Repeated, ongoing inhalation of LPS may result in habituation or tolerance (see later) with fewer neutrophils within airways. However, tolerance may be overcome by very large increases in endotoxin exposure or by some other agent interfering with defence mechanisms, a situation particularly relevant in equine environments in which endotoxin exposures can be highly variable.

1.4.5.1.2. Environmental versus Experimental Exposures

The minimum concentrations of total and respirable airborne endotoxin that cause airway inflammation and dysfunction have not been accurately defined for any species. Activation of macrophages and induction of cytokine production with subsequent lower airway inflammation may occur at inhalation doses as low as 0.5 to 5 µg of LPS in humans. However, endotoxin concentrations in airborne dust measured over an 8-hour/5-day week must generally be above 4.5 to 10 ng/m³ for detectable airway inflammation to occur in humans and be substantially higher to induce general respiratory symptoms and disease (100 to 200 ng/m³). Although these “no-effect levels” frequently are found in human occupational settings as well as in horse stable environments, they cannot be used as guidelines for horses due to extreme differences in duration and types of exposure.

“No-effect levels” have been estimated for healthy horses. As in humans however, doses of acutely inhaled LPS that induce airway inflammation (20 to 200 µg) greatly exceed the concentration of “endotoxin” present in organic dusts under conventional stable conditions that also initiates airway inflammation. This discrepancy may be due to the bioavailability of endotoxin or presence of other agents such as moulds and glucans in organic dust, longer duration of organic dust exposures and underestimation of biologically active endotoxin content in dust due to method of analysis. Importantly, these factors emphasise the difficulty with interpretation of, and measurement limitations of, environmental endotoxin exposures, and values quantified probably should be considered rough estimates only.

1.4.5.1.3. Endotoxin Tolerance

Tolerance to endotoxin is a poorly understood, adaptive and regulated response in animals involving down-regulation of macrophage activation as a result of repetitive LPS stimulation. The mechanisms responsible for down-regulation, while unclear, may involve alterations in LPS-dependent signal transduction pathways. Alternately, exposure to LPS or endotoxin can induce production of LPS-neutralising compounds. These compounds may block binding of active LPS to its receptors during a second exposure and may therefore be either primarily responsible for LPS hypo-responsiveness, or augment “LPS-tolerance” mechanisms that result in hypo-responsiveness to repetitive endotoxin exposures. Tolerance or hypo-responsiveness to endotoxin may also be the result of genetic factors.

In the early phase of tolerance, macrophages and monocytes exposed to LPS (experimentally) or endotoxin (in humans with sepsis) for 3 to 24 hours are rendered “tolerant” and when re-exposed to LPS or endotoxin manifest inhibited inflammatory mediator production. This effect may be transient if LPS or endotoxin exposure is removed, with recovery of macrophage responsiveness to endotoxin several days to weeks later. In contrast, late phase endotoxin tolerance is associated with development of
specific antibodies against LPS O-specific chain one to two weeks after initial endotoxin challenge and may last for months depending on the antigenicity of the endotoxin.

Tolerance or hypo-responsiveness to endotoxin may partly explain considerable inter-individual variability of response to inhaled endotoxin in humans. Variability in response most likely occurs in horses as well, although both humans and horses are known to be highly sensitive to the effects of endotoxin. Synergism between endotoxin and other inhaled infectious or non-infectious dust agents, particularly aeroallergens, undoubtedly also contribute to this variability in response to inhaled endotoxin in individuals.

Recent studies in healthy horses in which chronic intravenous infusion of endotoxin was performed demonstrate decreases in responsiveness to endotoxin and this response may also be observed in horses with clinical conditions associated with endotoxin exposure. It is feasible that endotoxin tolerance may also be a feature of horses exposed to endotoxin via inhalation of organic dusts but no solid evidence for such a response has yet been investigated.

1.4.5.1.4. Issues Regarding Measurement of Endotoxin

In the past, absolute concentrations of LPS were measured using elaborate and non-sensitive LPS extraction procedures including electrophoresis techniques, gas chromatography-mass spectrometry and high performance liquid chromatography. However, an in vitro biologic assay based on the reaction of Limulus amoebocyte lysate (LAL) with endotoxin is now widely used, particularly for analysis of particle aerosols. Importantly, while several modifications on the original LAL test have been developed, the quantitative end-point chromogenic modification is currently recommended for use as the standard test because of its high accuracy and reproducibility. This assay is used in this report. While an in depth discussion of the theoretical details behind the LAL test is given elsewhere, a brief outline of the concepts, and some methodological issues that may limit the validity and interpretation of the analyses relevant to this report, will be mentioned here.

The LAL test is derived from amoebocytes within the hemolymph of the horseshoe crab, Limulus polyphemus and provides an estimate of biological activity rather than the amount of LPS physically present. The cytoplasmic granules of the amoebocytes contain coagulation enzymes, which are activated by minute quantities (picograms) of endotoxin. Therefore, when amoebocytes, coupled with a synthetic dye or chromogenic substrate within the reagent in the LAL test, are mixed with endotoxin-containing samples (particles on filters or a liquid) a clotting cascade is activated. The rate-limiting step in the cascade is activation of factor C by endotoxin, with the rate of activation of this step directly related to the concentration of endotoxin present in the sample. The activated enzymes subsequently split para-nitroaniline (pNA) from the chromogenic substrate, which results in a yellow colour change that can be measured spectrophotometrically. The colour change is compared with colour changes from known standard endotoxin concentrations, providing a highly sensitive means of detecting amounts of endotoxin down to picogram levels in environmental samples. The unit of measurement of endotoxin is endotoxin units per millilitre of solution (EU/mL), with ~10-12 EU equal to 1 nanogram (ng) of endotoxin.

The LAL test may be subject to interferences causing inhibition or enhancement of endotoxin concentrations, rendering the test less valid under different conditions. The principle source of interference for dust samples collected on filters include non-specific LAL activation due to certain filter materials (eg., saponified cellulose), proteases and other products of Gram-negative and Gram-positive organisms and fungi within the sample. It is important to note that in addition to factor C, sensitive to endotoxin, the amoebocyte also contains factor G, which reacts with (1→3)-β-D-glucan. Although several commercial LAL assays contain both factor C and G and therefore are not specific for
endotoxin, cross-reactivity with glucan is believed to be negligible (Rylander, 2001 pers comm). However, endotoxin specific assays without factor G are commercially available 304,305. In addition, although results within one laboratory are highly reproducible, variations in results between laboratories are considerable (up to 1000 times), largely because of use of different protocols for extraction and handling, and use of different lots of LAL reagent 155,241,306. This situation emphasises the need for rigorous standardisation of the endotoxin testing in future so that valid comparisons of results can be made between studies using different laboratories. Finally, the results of the LAL test made on a dust sample, represent only about a third of biologically active endotoxin, the remainder present inside fragments of particles/bacterial cells but still able to exert effects when deposited within airways. Therefore, amounts detected in the analysis of dust should be multiplied by a correction factor of 3 to estimate the bioactive amount 241,279. In contrast, if endotoxin is present in a water solution, the measured value represents all of the endotoxin present in the sample.

1.4.5.2. (1→3)-β-D-Glucan
Mould exposure is a major environmental factor implicated in a variety of respiratory disorders and it is well recognised that their antigens have the capacity to induce an allergic IgE-related response in airways of humans 307 and horses 143. However, in recent years it has become increasingly clear that an important effect of moulds in humans is a non-specific airways inflammation as a result of the presence of glucans in their cell walls 147.

Glucans are widely distributed throughout nature in the cell wall of fungi or moulds, yeasts, certain bacteria and plants (eg., cereal grasses) 308. They are D-glucose polymers connected by α or β inter-chain linkages consisting of 1→3, 1→4 or 1→6 bonds 309. The (1→3) β-linked glucans (eg., grifolan) are the predominant form found in fungi 310 and most commonly exist as a stable complex of 3 polymer strands forming a triple helix 311 that primarily serves to maintain rigidity and integrity of the fungal cell wall 310. (1→3)-β-D-glucans are found in occupational (eg., waste composting facilities, and swine buildings) as well as in home environments 312. However, they are likely to be present in most indoor and outdoor environments contaminated with organic dust matter 312, such as horse stables, or in damp or humid environments in which appreciable mould growth is present. While research has focused on the immuno-biological effects of fungal (1→3)-β-D-glucan, other forms of glucans, particularly those derived from plants, also exert important biological effects 312 and would be present in variably high concentrations in organic dust contaminated environments. In addition, different fungi may contain varying amounts of glucan and they retain their effect even after death of the organism 313. It is important to also note that variable structure of glucans as well as the dose, route and animal model used in experimentation can significantly affect the biological activity of glucan 314,315, which probably explains why much discrepancy exists in literature regarding glucan actions and makes generalisation difficult.

(1→3)-β-D-glucan (referred to as “glucan” henceforth) can initiate non-specific inflammatory reactions and a variety of biological responses in animals and man. In vivo use produces host-mediated anti-tumour activity 316, enhances defence against bacterial challenge 317, increases haemopoeitic activity 318 and improves intestinal wound healing 319. In vitro, these molecules stimulate the mononuclear phagocyte system (MPS) 320, induce arachidonic acid metabolism 321, activate neutrophils 322, compliment 323, eosinophils 324 and macrophages, with release of specific inflammatory mediators 325,326 and nitric oxide 325,327.
Knowledge about the inflammatory effects of airborne glucan exposure in humans is limited and in some instances conflicts with *in vitro* findings. Currently, no information is available in horses. Information is derived mostly from acute and chronic exposures to different forms of glucan in laboratory guinea pigs and mice 235,268,328, which may or may not be extrapolated to horses or people. For example, acute inhalation of glucan alone at doses similar to concentrations found in the environment does not induce neutrophilic airway inflammation and if inhaled concomitantly with endotoxin, prevents endotoxin-stimulated invasion of neutrophils into airways 268. Although contradicting *in vitro* findings, the mechanism for the latter effect likely involves suppression of macrophage function, the discrepancy most likely due to differences in doses of glucan used and type of experimental model. In contrast, long-term exposure to glucan and endotoxin together enhances neutrophil invasion into airways 235, although this effect is not present with inhalation of glucan with a different helical structure 328. In addition, chronic exposure to glucan at quantities similar to environmental concentrations may induce eosinophilic airway inflammation 329, which is decreased by simultaneous exposure to endotoxin 328,330. The former effect may indicate that glucan induces a Th-2 inflammatory response, whereas the latter effect suggests inhibition of this response by a more potent Th-1 response induced by endotoxin-stimulated macrophages 328.

In reality, chronic simultaneous exposures to variable concentrations of glucan and endotoxin (and other pro-inflammatory agents) would probably occur in most environments contaminated with organic dusts, particularly horse stables. In general, glucans probably act primarily as adjuvants, exerting synergistic or additive effects when combined with other agents, or altering the susceptibility to other environmental agents, particularly allergens 336. Additionally, glucan concentrations found in various environments, plant materials or organic dusts are good indicators of total fungal content 331,332, which may be a valid means of determining degree of contamination of horse stables and feed sources and the likely risk of exposure for racehorses.

Analysis of (1→3)-β-D-glucan usually is performed using techniques that rely on the reactivity of the glucan molecule with factor G present in the amebocyte of the *Limulus* horseshoe crab. This method, although expensive, is very sensitive and reasonably specific for detection of (1→3)-β-D-glucan although it can react with other glucans. It is important to note that the triple helix configuration of (1→3)-β-D-glucan is not water-soluble and for accurate determination of glucan concentrations in environmental samples, must be rendered water-soluble before analysis using alkaline or heat treatment 238,333. Another less expensive although less sensitive method for quantification of (1→3)-β-D-glucans is an inhibition enzyme immunoassay 334. Similar issues regarding the limitations of use of the endotoxin *Limulus* assay described previously also applies to the glucan assay.
2. General Introduction and Materials and Methods

2.1 General Introduction

There is still a lack of information about how frequently IAD occurs in racehorse populations and of the factors that influence or determine this important respiratory disorder. Specifically, the various risk or causal factors, the sequence of clinical and pathophysiological events, the frequency, and the effect on performance in racehorses of IAD are still largely unknown, particularly in the Australian environment. The importance of improving our understanding of the distribution and determinants of lower airway inflammation in racehorses is underlined by the fact that respiratory disease, after lameness, is the most important cause of wastage or loss in the Thoroughbred racing industry, in Australia and overseas. In an industry estimated to contribute approximately $0.78 billion to Australian gross domestic product, this wastage not only represents a substantial economic loss but also has important welfare implications. Therefore, in the following report an attempt is made to characterise the epidemiology of IAD in young Australian racehorses, affording an opportunity for development of interventions aimed at reducing or preventing airway inflammation and disease, and for improving the welfare of racehorses in general.

One problem with clarifying these issues related to IAD is that, similar to the term “respiratory disease”, this inflammatory syndrome broadly encompasses many infectious and non-infectious, clinical and sub-clinical conditions of the LRT. In addition, researchers continue to debate whether IAD is a distinct disease or whether lower airway inflammation is part of a sequence of initiating environmental, allergic or infectious events, which may progress to more clinically recognisable disease endpoints. These endpoints might include non-infectious IAD, hyper-responsive bronchiolitis, infectious IAD and not just recurrent airway obstruction as has been advocated. Thus, to explore some of these clinical, aetiological and temporal issues, we carefully designed a large population-based study that would follow horses over time and allow periodic measurement of lower airway health and a range of factors related to that health.

With the increasing use of fiberoptic endoscopy to visualise tracheal mucopus and of guarded catheters to collect TA samples to characterise proximal lower airway cytology and bacteriology, IAD is now recognised as a serious health, performance-limiting and economic issue within the racing industry worldwide. Presence of lower airway inflammation may be characterised by increased proportions of neutrophils or eosinophils or both, or rarely, increased proportions of lymphocytes or mast cells. However, airway accumulations of mucus and/or neutrophils are the most common and most researched form of IAD found in young racehorses. Using increased quantities of mucopus visualised via tracheal endoscopy as a marker of airway inflammation, 22% to 50% of Standardbred or Thoroughbred racehorses in the UK and USA have been estimated as having IAD. In contrast, the prevalence of IAD in racehorses in the UK and USA defined using increased proportions of neutrophils (>20%) in TA samples has been estimated to be 14%, 27% and 33%. In Australia, the prevalence and incidence of neutrophilic IAD in young racehorses is unknown.

The cause of IAD currently is contentious and several non-infectious and infectious particulate agents acting sequentially or simultaneously probably are responsible for the development and/or maintenance of lower airway inflammation. Recently, longitudinal, case-control and retrospective studies focussing on viral and bacterial agents demonstrated the crucial significance of bacteria and their association with
neutrophilic airway inflammation\textsuperscript{4,8-11}. In addition, these studies highlighted the lack of association of IAD with common equine respiratory viruses\textsuperscript{4,8,11}. However, these studies also underlined the importance of non-infectious stable environment and management factors in producing airway inflammation in the high proportion of racehorses in which bacteria were not cultured, and that no studies to date have evaluated the inter-relationships and chronology of both non-infectious and infectious factors leading to lower airway inflammation.

The strong association between exposure to non-infectious particulate agents within air and inflammatory respiratory disease in humans\textsuperscript{155-157} and production animals\textsuperscript{158-160} has been documented for decades. In addition, overwhelming lines of evidence indicate that endotoxin along with (1→3)-β-D-glucan, fundamental components of organic particulate matter, when inhaled, contribute to acute and chronic airway inflammation and airflow obstruction in humans. Similar exposures to organic non-infectious dusts have been suggested as playing a significant role in the genesis, severity and duration of airway inflammation in stabled horses as well\textsuperscript{7,126,161,162}. Recent evidence also indicates that inhaled endotoxin, similar to the situation in humans and production animals, plays a vital role in development of acute airway inflammation in exposed horses housed in stables and probably in the development of RAO, a chronic respiratory disease observed in horses in Europe and North America\textsuperscript{128}. However, no scientific evaluation of these issues has been performed, particularly in Australian racetrack stable environments. The need for such studies is tellingly confirmed by the fact that even though Australian veterinarians believe that (after viruses) airborne dust is the most important risk for respiratory disease, this has not resulted in widespread use of low dust materials for bedding, in the practice of wetting down feed, or in improved ventilation standards in stables\textsuperscript{343}.

In view of the complex and multi-layered nature of IAD in racehorses, which has contributed to the paucity of information worldwide, a large, prospective cohort study was designed to better elucidate the distribution and determinants of this confusing equine respiratory disorder in young thoroughbred racehorses in Sydney. The broad aims of this cohort study were to:

1. Investigate as many non-infectious stable management and environmental risk factors, along with specific infectious agents, and determine their likely interactions, prevalence and their association with lower airway inflammation.
2. Calculate the incidence of acute subclinical neutrophilic airway inflammation or IAD as defined on the basis of TA cytology.
3. Examine the temporality of risk factors that precede the onset of airway inflammation, thus exploring possible causal links between these factors and lower airway inflammation.

Further to investigating primarily non-infectious environmental factors in the cohort study, the relationship between dust endotoxin and (1→3)-β-D-glucan and neutrophilic lower airway inflammation was investigated in a case-control study nested within the cohort study. In this study, only cases and randomly selected controls identified during the cohort study were investigated and from which samples of airborne dusts were collected. The specific aim of this case-control study was to:

1. Determine the association between respirable and total endotoxin and (1→3)-β-D-glucan in dusts in the breathing zone of young Thoroughbred racehorses housed in Sydney metropolitan racetrack stables, and two separate outcomes:
   a. The change in neutrophil percentage in TA samples.
   b. The occurrence of IAD, defined as > 20% neutrophils in TA samples.
2.2 Study Design

2.2.1 Preliminary Information
Three major metropolitan racetracks in Sydney were chosen to represent a defined geographical region and population of young racehorses. As many veterinarians as possible that serviced these tracks were contacted. Veterinarians were asked a number of broad preliminary questions related to their experience with IAD and about the management and training routine of the trainers to which they gave veterinary assistance. This ensured that relevant information provided by veterinarians was included in the final questionnaire/information sheet used in the study, increasing the quality and relevance of measurements while dispensing with less useful issues. Through liaison with track veterinarians, contact was subsequently made with as many racehorse trainers as possible.

2.2.2 Reference Population
Horses eligible for this study were 18 months to 42 months old (rising 2 to 3½ year old) Thoroughbred racehorses entering racetrack stables for a 6-10 week training schedule. This age group of horses were selected because of their greater propensity for developing lower airway inflammatory diseases than older horses \(^9,11,12,140,161\) and because they were a naïve, clinically healthy population entering stables for the first time.

These horses were not randomly sampled. Rather as many horses of this age as possible that were entering racetrack stables for training were included until as near to or greater than the required number of horses, was obtained. Eligible horses had no overt clinical signs of disease at time of entry and must have been in an outdoor paddock or yard for at least 2 weeks prior to entry. To maximise horse numbers, trainers were routinely contacted by phone or visited on Monday of every week ensuring that arrivals over the weekend were not missed.

The study was conducted between March 1999 to December 2001 (approximately 18 months) after which economic and time restrictions necessitated discontinuation. The major design used was a concurrent or prospective cohort study and is depicted in Figure 2.1. A case-control study nested within the cohort design also was performed.

2.2.3 Flow of Events
Data was collected from eligible horses at least 1 day after arrival at racetrack stables (designated “Day 0”) from pre-training or spelling establishments. This was to offset the possible effect on airway health of transport to the stable, in which distances of 0 to 200 or more km might have been travelled. However, horse data was collected within 3 days of arrival to ensure it was unaffected by the stable environment. Once placed within stables at baseline horses were subsequently “exposed to” different stable and loosebox environments and management, and then were monitored every 2 weeks for 4 weeks during their preparation for racing. The same data that was collected on Day 0 was also collected on Days 14 and 28, in particular to determine whether or not lower airway inflammation (the primary study “outcome”) had developed. This design consequently allowed exploration of a large number of variables, their interactions and their association with the study outcome. As well, data was collected at these time points because it was considered that 2 weeks was needed to allow effects of “exposures” to occur and outcomes to be manifest. In addition, these time points were used to allow adequate washout from the previous TA sample, to avoid imposing on trainers too frequently, for financial reasons and to minimise losses of horses, particularly as training neared completion beyond 4 to 6 weeks and risk of injury or movement from stables was higher. Within this design, selected horses were classified as cases or controls (as defined below) and samples of airborne respirable and total dusts, ammonia (NH\(_3\)) and carbon dioxide (CO\(_2\)) were collected from these horses only.
If management changed significantly during the study period, such as a change in type of bedding, movement of a horse to an alternate loosebox (eg., due to a physical or behavioural disorder) or if commencement of anti-inflammatory (eg., glucocorticosteroid) treatment occurred, then these horses were excluded from the study.

2.2.4 Definition for Inflammatory Airway Disease

The sole criterion used in this thesis to define lower airway inflammation or IAD was based on results of cytological examination of smears from TA samples. Horses with TA samples in which there was >20% neutrophils but ≤5% eosinophils were classified as having neutrophilic IAD. This definition was independent of any visual endoscopic findings such as URT discharge, pharyngeal lymphoid hyperplasia (PLH), tracheal oedema or erythema or tracheal mucus accumulation. However, IAD was also classified as eosinophilic if there was a high proportion of eosinophils (>5%) in the absence of >20% neutrophils. In addition, if both eosinophilic (>5%) and neutrophilic (>20%) components occurred together these cytological findings were classified as mixed IAD.
COHORT STUDY WITH NESTED CASE CONTROL STUDY

COHORT STUDY

STUDY POPULATION

DAY 0

Categorisation into groups by EXPOSURE to different study factors (eg., different stable environments)

DAY 14 AND 28

Develop OUTCOME

Do not develop OUTCOME

CASE CONTROL STUDY

“Cases”

Subgroup selected as “Controls”

Figure 2.1: Outline of cohort study design with nested case-control study.
2.2.5 Case and Control Definition and Selection

Horses were classified as **cases** if they had a cytological diagnosis of IAD as defined above, i.e., if cytological examination of smears from a TA sample demonstrated > 20% neutrophils (and ≤ 5% eosinophils) on differential count of at least 300 inflammatory cells. Horses were classified as cases after 14 or 28 days of being stabled at the racetrack stables and only if they were free of IAD when they entered racetrack stables at Day 0. Horses could be classified as a case once only.

Horses were classified as **controls** if cytological examination of smears from a TA sample demonstrated ≤ 20% neutrophils and ≤ 5% eosinophils and if horses were found on physical examination to be normal. At least 1 control per case was selected. A control horse could not have had IAD (i.e., been a case) at any time previously but may have been a control previously. Control horses were selected at the same time as cases were identified to ensure that the time during which a horse was eligible to be a control was the same time in which a horse was also eligible to become a case (if IAD should occur). In general, control horses were randomly selected from any yard in the racetrack stable area.

2.3 Data Collection

The author was solely responsible for all data collection, which was hand written onto prepared information sheets at the time horses were examined. A small number of specific questions were asked to identify prior and current historical issues about the horse, which may have been potential risk factors for disease, particularly at Day 0.

Most of the data collected were based on objective observations by the author and simplistic categories for each variable were used. Only variables that were hypothesised to have some biological or practical association with IAD or other outcome of interest were investigated. The same information was gathered on all horses at each time point in the cohort study and regardless of whether they became a case or control. The information collection sheet was divided into the following sections:

1. **Horse Details** (e.g., age, sex, behaviour in loosebox).
2. **Previous and Current History** (e.g., previous location, time and distance travelled, time since last worked, vaccination history, etc).
3. **Physical Examination** (e.g., heart and respiratory rates, temperature, head lymph node enlargement).
4. **Management Information** (e.g., bedding type, age and quality, feed type, ventilation/box design, fans, CO₂ concentration).
5. **Meteorological Information** (e.g., pollution index, ambient temperature, humidity, evaporation, wind).
6. **Endoscopic Information** (e.g., PLH, tracheal discharge grade and character).
7. **Tracheal Aspirate Information** (e.g., difficulty of collection, sample quality, cough during procedure).

2.4 Sample Collection

Samples of blood for serological analysis and tracheal aspirate samples were collected on all horses during the study whereas airborne dust samples and samples of ammonia and carbon dioxide were obtained only from selected horses recruited for the case-control study.

2.4.1 Blood Collection

Paired blood samples were collected 14 days apart into plain vacuum tubes using a standard technique and stored on ice (for approximately 2 hours) during transport to the laboratory. Serum was separated by centrifugation of tubes at 10 000 rpm for 5 minutes. Two mL aliquots of serum were stored at -20°C until batch analysis for serological analysis for Equine Herpes virus (EHV)-1 and -4, equine adenovirus, equine influenza virus 1 and 2, and equine rhinitis virus (ERV) A and B was performed using techniques
previously described. All samples were sent to laboratories at the Animal Health Trust in Newmarket, England, UK where researchers have extensive expertise with viral serological analysis and interpretation. Complement fixation tests were used for EHV-1 and -4 and ER-A and –B, using methods derived from those of Thomson et al (1976). The haemagglutination inhibition test was used to measure antibodies to equine influenza and adenovirus using methods described by Powell (1991). Seroconversion to any virus was indicated by a four-fold increase in antibody titre. Horses were also classified on the basis of their initial antibody titre. For EHV, horses with no detectable complement fixation antibody were considered as having no evidence of previous infection. Horses with antibody titres greater than 0 but ≤ 80 were considered to have evidence of exposure whereas those with initial titres > 80 were considered to have evidence of recent infection. For ERV-A and -B, initial titres were classified as either zero, suggesting no previous infection, or greater than zero, suggesting recent infection (JLN Wood, pers comm). For equine adenovirus and influenza initial titres were classified as zero (suggesting no previous exposure), greater than zero but ≤ 32 (suggesting previous exposure), or > 32 (suggesting recent exposure; JLN Wood pers comm).

2.4.2 Collection of Tracheal Aspirate Samples
Tracheal aspirates were collected using a standard endoscopic technique and using a guarded catheter. All TA samples were collected from horses standing in their looseboxes at racetrack stables.

Horses were restrained using a nose twitch. The external surface and instrument channel of the endoscope (Olympus CF type 20L, 1.8 metre fibreoptic endoscope, Tokyo, Japan) was disinfected then flushed with copious quantities of distilled water, with the excess fluid blown out of the channel using an air-filled syringe. A Darien Microbiology Aspiration Catheter (DMCA; Bard Interventional/Mill Rose Inc, Mentor, Ohio, USA) was introduced into the instrument channel of the endoscope. The endoscope was passed via the left nostril, through the rima glottidis and advanced down the trachea until the dorsal curvature of the distal trachea was reached at the carina. The catheter was passed from the instrument port into the tracheal lumen and the 2 inner guarded catheters were quickly introduced into the trachea. Ten mL of sterile saline was then instilled, followed immediately by suction of the fluid back into the syringe. When 8-10 mL of fluid was retrieved, suction was stopped, the catheter was replaced within the biopsy channel and the endoscope removed from the respiratory tract. In the majority of cases, this amount of fluid was recovered for analysis, while the entire procedure would take approximately 2-3 minutes. The syringe containing the tracheal aspirate was placed on ice and transported to the laboratory within 1-3 hours of collection. Details of the procedure, including grade of tracheal mucus, quantity and gross quality of the fluid sample collected were recorded on prepared sheets.

2.4.2.1 Laboratory Analysis of Tracheal Aspirate Samples
Approximately 1.5 to 2 mL of the TA sample was transferred in a sterile manner into a sterile 2 mL vial for bacteriological analysis. The remainder of the sample (usually > 3 mL) was placed into a labelled non-sterile container for cytological analysis.

2.4.2.1.1 Cytological Evaluation
The TA fluid was mixed by gentle inversion of the container and attempt was made to loosen and disperse cell-containing mucus aggregations by gently flicking the side of the container. One, 500 µL aliquot of TA fluid was cytocentrifuged (Cyto-tek Centrifuge, Miles Scientific, Sakura Finetechanical Co. Japan) onto a labelled clean glass slide. Slides were air-dried and subsequently stained with Diff-Quik® (Baxter, Illinois, USA). After blow-drying, smears were carefully scrutinised for a range of cytological variables, initially at 100×, then under oil immersion (1000×) microscopy.
Each slide was evaluated for quantity of mucus, proportions of neutrophils, lymphocytes, eosinophils, mast cells, macrophages, haemosiderophages, giant cells, macrophages ingesting debris, squamous epithelial cells, ciliated columnar epithelial cells, cuboidal epithelial cells, free erythrocytes, and Curshmann’s spirals of mucus. Differential counts of 100 inflammatory cells were made in each of 3 to 6 separate sections of the smear, using high power (1000×) light microscopy. In particular, the mean of the 3 to 6 neutrophil counts, expressed as a percentage of the total number of inflammatory cells counted, was then categorised as either above or below a cut-off value of 20%. Other categories of inflammatory airway disease also were determined if percentage of eosinophils was >5% or if there was a combination of >20% neutrophils and >5% eosinophils.

Smears were considered adequate only if they demonstrated satisfactory numbers of cells from all levels of the pulmonary tree, including ciliated columnar epithelial cells, cuboidal epithelial cells and alveolar macrophages. Smears from samples that had few or no epithelial cells or macrophages or with <100 inflammatory cells in total were considered the result of poor retrieval of tracheal secretions and were considered uninterpretable.

2.4.2.1.2 Bacteriological Evaluation

The number of colony forming units per millilitre (cfu/mL) of TA sample was assessed using aerobic bacterial cultures only. Anaerobic cultures were not performed because previous studies in similar populations of racehorses demonstrated that anaerobic bacteria were not associated with IAD or with clinical signs of IAD (ie., cough)\(^6\) and therefore would probably be of limited importance in this study.

Numbers of cfu/mL were calculated by counting the cfu on plates. Identification of colonies was only performed if the number of cfu/mL was \( \geq 10^3 \)\(^4\) and was based on standard techniques, including reaction to gram staining and biochemical tests\(^3\). For the purposes of analysis, quantitative bacterial counts were categorised as either significant growth (cfu/mL \( \geq 10^3 \)) or not. In addition, the change in significant bacterial growth over 2 weeks was determined as a means to investigate any association between this change and development of IAD. Bacterial growth was categorised as insignificant or had cleared over 2 weeks, or had maintained or developed over 2 weeks.

2.4.3 Carbon Dioxide and Ammonia Determination

Ammonia and carbon dioxide was measured using a portable, hand-held, direct-reading air analyser (Dräger CMS\(^6\), Lübeck, Germany). Measurement chips or gas-specific capillaries (specific for CO\(_2\) [range: 200-3000 ppm] or NH\(_3\) [range: 0-50 ppm]), which were temperature corrected and calibrated, were inserted into the analyser. When switched on after automatic calibration, the analyser drew air at a constant rate through the gas-specific reagent system encapsulated in the capillaries. The gas to be measured (either CO\(_2\) or NH\(_3\)) induced a chemical reaction, which was detected optoelectronically. The analyser was held approximately 1 metre above the ground at various points inside the loosebox in which the selected horse (ie., a case or control) was housed. Thirty to sixty seconds was taken for each measurement and the concentration of gas in units of ppm was displayed in digital format. These measurements were used as proxy or surrogate variables, CO\(_2\) as a reflection of ventilation quality of the loosebox and NH\(_3\) as a reflection of ventilation quality and exposure to this potentially noxious gas in the loosebox on a day-to-day basis. Concentrations of CO\(_2\) were divided into quartile categories whereas NH\(_3\) concentrations were categorised as < 2 ppm or \( \geq 2 \) ppm.

2.4.4 Collection of Airborne Dust Samples

Personal Air Samplers (PAS) were used to collect dust onto 25 mm diameter, 0.8µm pore size polycarbonate filters (Millipore, Bedford, MA USA) using guidelines supplied by Standards Australia\(^2\). The PAS consisted of a programmable pump (224-PCXR8, Airmet Scientific Pty Ltd, Nunawading, Australia) to create a constant vacuum source, the filter, a filter holder and coiled latex rubber tubing.
attaching pump to filter holder. For dust measurements a cyclone impactor (Higgins Cyclonic Impactors, Casella, London; 214,348) was used.

On the day of sampling, using chemically sterilised forceps, filters were carefully placed and secured tightly into filter holders, preventing leakage of air from around the filter edge. The vacuum pumps were programmed to run for approximately 5 or more hours at a flow rate of 2.0 ± 0.1 L/minute 214,349. This time (between 9.30am and 2.30pm) was selected to allow convenient collection of an adequate and representative dust sample when horses invariably were eating and some form of human activity was taking place inside and sometime outside the loosebox (eg., removal of faeces and urine from bedding or laneway sweeping).

Pumps were wrapped in plastic bags and two were taped securely and as high as possible on either side of a surcingle. The surcingle was buckled just behind a horse’s wither to reduce risk of damage or interference from the horse and allowed the horse to move freely at all times. The cyclone impactor was taped on either side of the horse’s head collar approximately 15cm away from the nostrils, well within the breathing zone (30cm) of the horse 214. At the conclusion of the sampling period, filters were carefully removed from impactors, avoiding dislodgement of dust from the filter, and placed into sterile, pre-labelled micro petri dishes and sealed using parafilm (Parafilm “M” Laboratory film, American National CanTM, Greenwich, USA).

The sealed filters within the petri dishes were stored at -4°C in a lightless commercial fridge, in order to prevent growth of additional micro-organisms that might contribute to the endotoxin, (1→3)-β-d-glucan or assay-reactive material load of the sample 298. Analyses for endotoxin and (1→3)-β-D-glucan were performed on all filters at the same time at the completion of the study. This was done at a specialist laboratory at the University of Edinburgh, Scotland using a commercially available endotoxin-specific assay (Endospecy, Seikagaku Co., Tokyo, Japan) 304 and an (1→3)-β-D-glucan-specific assay (Fungitec G, Seikagaku Co. Tokyo, Japan) 304.

2.5 Statistical Analysis

Data were recorded in computer databases (Microsoft Excel, MS Office 2000, Microsoft Corporation, USA). The entire data set from approximately 10% of randomly selected horses were crosschecked for internal consistency. All variables were reviewed to ensure analysability and biological plausibility.

2.4.1 Descriptive Statistics

All data were recorded in Excel spreadsheets (MS Office 2000, Microsoft Corporation, USA), checked for errors and descriptive statistics calculated in Excel or by importing relevant data into Minitab (Minitab Student 12.0). In general, numbers of horses with or without a particular risk factor between Day 0 and 14 were determined and the proportion of these horses with airway inflammation (ie., prevalence proportion) was calculated and expressed as a percentage with it’s 95% confidence interval (CI). Where necessary to emphasise a statistically significant (or near significant) difference, Chi squared ($\chi^2$) tests were used to compare prevalence of IAD between categories within a study variable (eg., 2-year-olds versus 3-year-olds). All results (for all analyses) were considered significant if P<0.05.

Cumulative incidence was defined as the sum of horses that developed neutrophilic IAD within the first 2 weeks of being stabled divided by the sum of horses for which data was available (ie., excluding horses lost to follow-up), over the entire 22 month study period. In contrast, Incidence Rate (IR) was calculated as the sum of all new cases occurring between Day 0 and Day 14 divided by the sum of “horses at risk” (excluding horses lost to follow-up and horses that developed some other outcome) in the first 2-week period of study. The “horse-fortnight”, representing each 14 days or fortnight that TA samples were
collected and horses were monitored, was used as the unit of observation. However, horses that developed IAD were assumed to have done so after about 1 week in stables and maintained inflammation until evaluated at Day 14 (and Day 28). Incidence Rate was calculated for the second fortnight of risk (from Day 14-Day 28) in a similar manner, dividing the sum of all new cases occurring between Day 14 and 28 by the sum of horses at risk in the second 2 week or fortnight period.

2.5.2 Detailed Analysis of All Data
In order to investigate (in the cohort study) as many non-infectious stable management and environmental risk factors, along with specific infectious agents, and determine their association with IAD, two statistical analyses were performed. One included only horses that were free of airway inflammation at day 0 (n=138) whereas the second included all horses regardless of their airway status at day 0 (n=235). This latter analysis therefore included as a variable “Day 0 airway status”, allowing investigation of whether or not airway inflammation in horses arriving at racetrack stables was associated with airway inflammation after 2 weeks in the stable (at day 14).

Up to 100 variables were screened using univariable regression analyses (SAS version 8.0, Australia). Only variables significantly associated with the outcome under investigation, and variables that were considered a priori to be important, were included in multivariable regression analyses. Therefore, analysis of all relevant risk factors together allowed examination of the effect of a specific risk factor while controlling the effects of all the others.

A similar statistical procedure was used to determine (in the case-control study) the effect of endotoxin and (1→3)-β-D-glucan in dust in the breathing zone of young racehorses on change in neutrophil percentage in TA samples and on occurrence of IAD. However, only horses selected as cases or controls were used in this analysis.

It is important to remember that whereas in the cohort study the outcome being investigated was whether or not horses developed IAD, in the case-control study two outcomes were evaluated, namely the change in percentage of neutrophils in TA and, as for the cohort study, development of IAD.
3. Results

3.1 Descriptive Statistics

Results of the number of horses and the prevalence (and 95% confidence interval where relevant) of those with lower airway inflammation are presented in Tables 3.1 to 3.5 and in Figures 3.1 to 3.3. It should be noted that emphasis was placed mostly on Day 0 and 14 and some Day 28 data of the cohort study. Between March 2000 to December 2001, data was collected from a total of 235 horses from Day 0. Nine trainers from three racetrack establishments were recruited during the study although only seven trainers remained at the end of the study, two lost as a result of problems with compliance with research criteria. Within the first 2 weeks, due to loss of 30 horses (13%) to follow-up, data was available for 205 (87%) horses at Day 14 (Table 3.1). Between Day 14 and 28, 45 more horses (22% [45/205]) were lost to follow-up and a total of 160 horses (68%) therefore completed the 4-week study (from Day 0 to Day 28). The major reason for horses leaving the study over 4 weeks was due either to moving to another box or, more rarely, travel to another stable for spelling (65 horses, 62% of which were moved between Day 14 and 28). Rarely, horses were lost as a result of musculoskeletal injury (9 horses) or due to behavioural problems (1 horse).

Table 3.1: General details of horse numbers and losses to follow-up over 4 weeks.

<table>
<thead>
<tr>
<th>Horse Details</th>
<th>Day 0 (Number [%])</th>
<th>Day 14 (Number lost [%])</th>
<th>Day 28 (Number lost [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>235 (100%)</td>
<td>30 (13%)</td>
<td>45 (22%)</td>
</tr>
<tr>
<td><strong>Signalment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 year old</td>
<td>172 (73%)</td>
<td>22 (13%)</td>
<td>38 (25%)</td>
</tr>
<tr>
<td>3 year old</td>
<td>63 (27%)</td>
<td>8 (13%)</td>
<td>7 (13%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colt</td>
<td>49 (21%)</td>
<td>5 (10%)</td>
<td>7 (16%)</td>
</tr>
<tr>
<td>Gelding</td>
<td>66 (28%)</td>
<td>9 (14%)</td>
<td>13 (23%)</td>
</tr>
<tr>
<td>Female</td>
<td>120 (51%)</td>
<td>16 (13%)</td>
<td>25 (24%)</td>
</tr>
<tr>
<td><strong>Trainer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>86 (37%)</td>
<td>10 (12%)</td>
<td>20 (26%)</td>
</tr>
<tr>
<td>2</td>
<td>36 (15%)</td>
<td>5 (14%)</td>
<td>6 (19%)</td>
</tr>
<tr>
<td>3</td>
<td>42 (18%)</td>
<td>2 (5%)</td>
<td>5 (13%)</td>
</tr>
<tr>
<td>4</td>
<td>31 (13%)</td>
<td>6 (19%)</td>
<td>6 (24%)</td>
</tr>
<tr>
<td>5</td>
<td>14 (6%)</td>
<td>3 (21%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>6</td>
<td>7 (3%)</td>
<td>2 (29%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>7</td>
<td>16 (7%)</td>
<td>1 (6%)</td>
<td>3 (20%)</td>
</tr>
<tr>
<td><strong>Stable area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>164 (70%)</td>
<td>17 (10%)</td>
<td>31 (21%)</td>
</tr>
<tr>
<td>2*</td>
<td>54 (23%)</td>
<td>12 (22%)</td>
<td>11 (26%)</td>
</tr>
<tr>
<td>3*</td>
<td>17 (7%)</td>
<td>1 (6%)</td>
<td>3 (19%)</td>
</tr>
</tbody>
</table>

* Results include 2 other trainers (contributing only 3 horses) that were dropped from study. Proportions of horses lost after Day 14 and 28 are calculated from total numbers available at the previous collection day.

More horses were lost between Day 14 and 28 with 12% more 2-year-old horses than 3-year-old horses lost and 7 to 8% more geldings and female horses respectively lost than colts in this 2-week period. Excluding trainer 5 who lost less horses over time, the median percentage of horses lost amongst trainers between Day 0 and 14 was 13% whereas the median percentage lost between Day 14 and 28 was 22%. Similarly, proportionately more horses were lost from each stable area between Day 14 and 28, with stable area 2 suffering greater losses than the other training establishments over the 4 weeks.
3.1.1 Incidence of Neutrophilic Lower Airway Inflammation or IAD

The cumulative incidence for neutrophilic airway inflammation in healthy young horses housed in racetrack stables for 2 weeks was 40.3% (95%CI: 31.5-49.2%). Of these horses, IAD continued for 2 further weeks in 50% (95% CI: 35-66%), whereas IAD resolved in 48% (95% CI: 32-63%) of horses (Table 3.2 and Figure 3.1). Horses that were IAD-free after 2 weeks in stables (52.1% [95% CI: 43.1%-61.1%]) largely remained healthy over the next 2 weeks with only 18% (95% CI: 7-28%) developing IAD. In addition, the cumulative incidence for eosinophilic or mixed airway inflammation in young horses housed in racetrack stables for 2 weeks was 5.0% and 1.6% respectively (Figure 3.1).

Table 3.2 and Figure 3.2 also highlight the large proportion of horses that had some form of IAD when they first arrived at racetrack stables (86/235 or 36.6% [95% CI: 30.4% - 42.8%]), the majority having neutrophilic IAD (56/86 or 65%). In general, regardless of the type of IAD horses had at Day 0, the same or alternate type of inflammation persisted in a small proportion of horses for 2 further, and often 4, weeks (Figure 3.2). In addition, of the 11 horses that could not be cytologically classified at Day 0 due to inadequate cell density on smears from TA samples, most (7) had no tracheal discharge and fluid retrieved from TA samples was always visually clear. After 2 weeks, approximately half of these horses still could not be cytologically classified (Figure 3.2).

To account for losses of horses during the study, Figure 3.3 illustrates the cumulative incidence of neutrophilic airway inflammation if no horses during the entire study were lost and instead developed, or did not develop, lower airway inflammation or “other”. This diagram indicates that the period prevalence for neutrophilic airway inflammation in young horses housed in racetrack stables for 2 weeks may have been as low as 35% or as high as 49%.

Using Figure 3.1, a total of 86 horse-fortnights of data were collected from 110 horses (138 horses free of neutrophilic IAD at Day 0 minus 19 lost to follow-up and minus 9 that developed some other form of IAD) over 22 months of research. This represented the first 2 weeks (or first fortnight) racehorses were housed in stables. Therefore, the Incidence Rate of neutrophilic lower airway inflammation was 48 cases per 86 horse-fortnight or 55.8 cases per 100 horses per fortnight of observation or risk. During the “second fortnight” (between Day 14 and 28) housed in stables, a total of 45.5 horse-fortnights of data were collected from 50 horses (62 horses free of neutrophilic IAD at Day 14 minus 11 lost to follow-up and minus 1 that developed some for of IAD). The Incidence Rate of neutrophilic lower airway inflammation was 9 cases per 45.5 horses-fortnight or 19.8 per 100 horses per fortnight of risk.

3.1.2 Horse Details

In general, a higher proportion of horses were found to be free of IAD if they were free of inflammation at the previous measurement day, particularly between Day 14 and 28 (Table 3.2). Also, a higher proportion of 2-year-old racehorses than 3-year-olds arrived at racetrack stables for training with neutrophilic airway inflammation (27% vs 16%; P=0.08) whereas more 3-year-old racehorses previously free of airway inflammation developed IAD after 2 (43% vs 39%) and 4 weeks (22% vs 15%) in stables. In addition, a greater proportion of IAD was found on arrival and after 2 weeks in stables in colts than either geldings or females (Table 3.2).
3.1.3 Management Information

Approximately 70% of trainers used sawdust bedding for looseboxes and most bedding was damp. Although numbers were low, a higher proportion of horses housed in looseboxes bedded with straw developed neutrophilic IAD than horses bedded on other types of bedding (80% vs 37%; P=0.05) (Table 3.3a).

Feed was mostly placed in containers held off the ground although similar proportions of horses developed neutrophilic IAD regardless of feed position. Although numbers were low, a higher proportion of horses fed greater quantities of oats and corn, than those fed an even quantity of chaff and oats or solely chaff, developed neutrophilic IAD (75% vs 30.6%-39.5%) (Table 3.3a).

Table 3.3a: Number and percentage of horses and the prevalence and 95% CI of neutrophilic IAD on Day 14, categorised by management factors at stables.

<table>
<thead>
<tr>
<th>Variable and Categories</th>
<th>No. of horses (%)</th>
<th>No. Neutrophilic IAD at Day 14 (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Management Info</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bedding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Straw</td>
<td>5 (2.4%)</td>
<td>4 (80.0%) (74.5% - 85.5%)</td>
</tr>
<tr>
<td>Sawdust</td>
<td>145 (70.7%)</td>
<td>53 (36.6%) (29.9% - 43.1%)</td>
</tr>
<tr>
<td>Shavings/sawdust</td>
<td>51 (24.9%)</td>
<td>19 (37.3%) (30.6% - 43.9%)</td>
</tr>
<tr>
<td>Sand</td>
<td>4 (1.9%)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Depth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-20cm</td>
<td>195 (95.1%)</td>
<td>72 (36.9%) (30.3% - 43.5%)</td>
</tr>
<tr>
<td>&gt;20cm</td>
<td>10 (4.9%)</td>
<td>4 (40.0%) (33.3% - 46.7%)</td>
</tr>
<tr>
<td><strong>Quality change</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry over 2 wks</td>
<td>6 (2.9%)</td>
<td>3 (50.0%) (43.2% - 56.8%)</td>
</tr>
<tr>
<td>Damp over 2 wks</td>
<td>184 (89.8%)</td>
<td>68 (36.9%) (30.4% - 43.6%)</td>
</tr>
<tr>
<td>Any change over 2 wks</td>
<td>15 (7.3%)</td>
<td>5 (33.3%) (26.9% - 39.8%)</td>
</tr>
<tr>
<td><strong>Age change</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium over 2 wks</td>
<td>81 (39.5%)</td>
<td>29 (35.8%) (29.2% - 42.4%)</td>
</tr>
<tr>
<td>Old over 2 wks</td>
<td>17 (8.3%)</td>
<td>7 (41.2%) (34.4% - 47.9%)</td>
</tr>
<tr>
<td>Older/topped up</td>
<td>88 (42.9%)</td>
<td>33 (37.5%) (30.9% - 44.1%)</td>
</tr>
<tr>
<td>Complete refresh</td>
<td>19 (9.3%)</td>
<td>7 (36.8%) (30.2% - 43.5%)</td>
</tr>
<tr>
<td><strong>Feed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay type</td>
<td>(n=189)*</td>
<td></td>
</tr>
<tr>
<td>Straw (high dust)</td>
<td>27 (14.3%)</td>
<td>11 (40.7%) (33.7% - 47.8%)</td>
</tr>
<tr>
<td>Lucerne (medium dust)</td>
<td>141 (74.6%)</td>
<td>53 (37.6%) (30.7% - 44.5%)</td>
</tr>
<tr>
<td>Clover (low dust)</td>
<td>21 (11.1%)</td>
<td>6 (28.6%) (22.1% - 35.0%)</td>
</tr>
<tr>
<td><strong>Feed off ground</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>151 (73.7%)</td>
<td>55 (36.4%) (29.8% - 43.0%)</td>
</tr>
<tr>
<td>No</td>
<td>54 (26.3%)</td>
<td>21 (38.9%) (32.2% - 45.6%)</td>
</tr>
<tr>
<td><strong>Relative proportions of hard feed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High chaff</td>
<td>129 (62.9%)</td>
<td>51 (39.5%) (32.8% - 46.2%)</td>
</tr>
<tr>
<td>Even chaff/oats</td>
<td>72 (35.1%)</td>
<td>22 (30.6%) (24.3% - 36.9%)</td>
</tr>
<tr>
<td>High oats/corn</td>
<td>4 (1.9%)</td>
<td>3 (75.0%) (69.1% - 80.9%)</td>
</tr>
</tbody>
</table>

* This variable only had less horses due to inability to classify hay type because hay was absent in loosebox.
Table 3.2: Number and prevalence of horses on Day 0, 14 and 28 free of lower airway inflammation that subsequently developed neutrophilic IAD at the next measurement day, categorised by age, sex, trainer and location of stable. Results are expressed as a percentage of the total number of horses available at the previous measurement day, accounting for losses after 14 and 28 days.

<table>
<thead>
<tr>
<th>Horse details</th>
<th>No. of horses (%)</th>
<th>No. free of airway inflammation (%)&lt;sup&gt;^&lt;/sup&gt;</th>
<th>No. with neutrophilic IAD (%)&lt;sup&gt;*&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>2 year old</td>
<td>172 (73%)</td>
<td>96 (56%)</td>
<td>43 (52%)</td>
</tr>
<tr>
<td>3 year old</td>
<td>63 (27%)</td>
<td>42 (67%)</td>
<td>19 (51%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>235 (100%)</td>
<td>138 (59%)</td>
<td>62 (52%)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colt</td>
<td>49 (21%)</td>
<td>23 (47%)</td>
<td>9 (43%)</td>
</tr>
<tr>
<td>Gelding</td>
<td>66 (28%)</td>
<td>42 (64%)</td>
<td>18 (51%)</td>
</tr>
<tr>
<td>Female</td>
<td>120 (51%)</td>
<td>73 (61%)</td>
<td>35 (56%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>235 (100%)</td>
<td>138 (59%)</td>
<td>62 (52%)</td>
</tr>
<tr>
<td><strong>Trainer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>86 (37%)</td>
<td>49 (57%)</td>
<td>28 (65%)</td>
</tr>
<tr>
<td>2</td>
<td>36 (15%)</td>
<td>21 (58%)</td>
<td>5 (28%)</td>
</tr>
<tr>
<td>3</td>
<td>42 (18%)</td>
<td>17 (40%)</td>
<td>8 (47%)</td>
</tr>
<tr>
<td>4</td>
<td>31 (13%)</td>
<td>22 (71%)</td>
<td>7 (39%)</td>
</tr>
<tr>
<td>5</td>
<td>16 (7%)</td>
<td>11 (69%)</td>
<td>4 (57%)</td>
</tr>
<tr>
<td>6</td>
<td>7 (3%)</td>
<td>5 (71%)</td>
<td>2 (67%)</td>
</tr>
<tr>
<td>7</td>
<td>17 (7%)</td>
<td>13 (76%)</td>
<td>8 (62%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>235 (100%)</td>
<td>138 (59%)</td>
<td>62 (52%)</td>
</tr>
<tr>
<td><strong>Stable Area</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>164 (70%)</td>
<td>87 (53%)</td>
<td>41 (53%)</td>
</tr>
<tr>
<td>2</td>
<td>54 (23%)</td>
<td>38 (70%)</td>
<td>13 (46%)</td>
</tr>
<tr>
<td>3</td>
<td>17 (7%)</td>
<td>13 (76%)</td>
<td>8 (62%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>235 (100%)</td>
<td>138 (59%)</td>
<td>62 (52%)</td>
</tr>
</tbody>
</table>

IAD: Inflammatory Airway Disease. CI: Confidence Interval.

<sup>^</sup> Indicates number of horses free of neutrophilic, eosinophilic or mixed forms of IAD expressed as a proportion of available horses at previous day of measurement. Results of other forms of IAD are illustrated in Figure 2.

<sup>*</sup> Assuming free of IAD at previous day of measurement. For example, of the 96 that were free of IAD at Day 0, 32 two-year-old racehorses developed IAD at Day 14 whereas 5 of the 43 two-year-old racehorses that were free of IAD at Day 14 developed IAD at Day 28.

NB. A total of 3 horses from 2 stables that were both dropped during study were included in their respective stable areas and added onto totals of trainers situated close by.
Figure 3.1: Flow diagram demonstrating fate of horses over 28 days (1 month) that were free of airway inflammation at Day 0. Results are expressed as numbers of horses, the percentage of total available horses and 95% CI of the percentage in parentheses.

* “Other” refers to horses with some form of IAD other than neutrophilic IAD or undetermined result.
### COHORT STUDY

#### DAY 0

2 and 3 year old TB horses: n=235

- 86 Some form of IAD: 58.7% (95% CI: 52.4-65.0%)
- 138 IAD-free: 36.6% (95% CI: 30.4-42.8%)
- 11 No result: 4.7% (95% CI: 2.0-7.4%)

#### NIAD

- 56 NIAD: 65% (95% CI: 55-75%)
- 20 EIAD: 23% (95% CI: 14-32%)
- 10 MIAD: 12% (95% CI: 5-18%)

#### DAY 14

- 7 lost
- 1 lost

### Day 28

<table>
<thead>
<tr>
<th></th>
<th>NIAD</th>
<th>NonIAD</th>
<th>EIAD</th>
<th>MIAD</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIAD</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NonIAD</td>
<td>7</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>EIAD</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MIAD</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NR</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Lost</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**Figure 3.2:** Flow diagram demonstrating cytological fate of all horses over 28 days (1 month) that had some form of airway inflammation at Day 0. Results are expressed as numbers of horses, the percentage of total available horses and where relevant the 95% CI of the percentage in parentheses. Results from Day 14 to Day 28 should be read downwards. For example, of 21 horses with NIAD at Day 14, at Day 28 6 developed NIAD, 7 resolved, 1 EIAD and 7 were lost to follow-up.

**KEY:** NIAD = Neutrophilic IAD; EIAD = Eosinophilic IAD; MIAD = mixed IAD; Non-IAD = IAD-free; NR = No result.
**COHORT STUDY**

**DAY 0**

- 2 and 3 year old TB horses: \( n = 235 \)
  - 138 IAD-free: 58.7%
  - 86 Some form of IAD: 36.6%
  - 11 No result: 4.7%

**DAY 14**

- 19 lost to follow-up
  - 9 or 28 Other*: (7% - 20%)
  - 62 or 81 IAD-free: (45% - 59%)
  - 48 or 67 developed IAD: (35% - 49%)

**DAY 28**

- 3 lost to follow-up
- 11 lost to follow-up
- 8 lost to follow-up
  - 41 or 52 remained IAD-free: (66% - 84%) or (51% - 64%)
  - 1 or 12 Other*: (15% - 32%) or (11% - 25%)
  - 20 or 28 continued IAD: (42% - 58%) or (30% - 42%)
  - 1 or 9 Other*: (15% - 32%) or (11% - 25%)

**Figure 3.3:** Flow diagram demonstrating fate of horses over 28 days (1 month) that were free of airway inflammation at Day 0. Results in parentheses show the prevalence of neutrophilic IAD if no horses were lost to follow-up and instead either developed IAD or did not develop IAD between Day 0 and 14 and between Day 14 and 28.

* “Other” refers to horses with some form of IAD other than neutrophilic IAD or undetermined result.
There was a reasonably even distribution of stables that utilised windows for ventilation although prevalence of neutrophilic IAD was almost 2-fold higher in horses housed in looseboxes with no windows than in horses housed in looseboxes with an open window (45.4% vs 27.1%; P=0.02) (Table 3.3b).

Subjectively, the majority of looseboxes had some form of “bedding” odour (~74%). However, the prevalence of neutrophilic IAD in horses housed in looseboxes in which no odour was subjectively detected was half that in horses housed in boxes in which “weak or strong ammonia” odour was detected. In addition, lower proportions of horses housed in looseboxes adjacent to laneways that were swept and washed down regularly rather than swept only developed neutrophilic IAD (30.9% vs 42.6%) (Table 3.3b).

Table 3.3b: Number and percentage of horses and the prevalence and 95% CI of neutrophilic IAD on Day 14, categorised by management factors at stables.

<table>
<thead>
<tr>
<th>Variable and Categories</th>
<th>No. of horses (%)</th>
<th>No. Neutrophilic IAD a Day 14 (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Management Info</strong></td>
<td><strong>n=205</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ventilation proxy variables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any open windows</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>86 (41.9%)</td>
<td>39 (45.4%) (38.5% - 52.2%)</td>
</tr>
<tr>
<td>Yes</td>
<td>70 (34.2%)</td>
<td>19 (27.1%) (21.1% - 33.2%)</td>
</tr>
<tr>
<td>Open air component</td>
<td>49 (23.9%)</td>
<td>18 (36.7%) (30.1% - 43.3%)</td>
</tr>
<tr>
<td>Presence of fans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes: on</td>
<td>17 (8.3%)</td>
<td>6 (35.3%) (28.8% - 41.8%)</td>
</tr>
<tr>
<td>No or yes but off</td>
<td>188 (91.7%)</td>
<td>70 (37.2%) (30.6% - 43.9%)</td>
</tr>
<tr>
<td>Incomplete walls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None or 1</td>
<td>47 (22.9%)</td>
<td>18 (38.3%) (31.6% - 44.9%)</td>
</tr>
<tr>
<td>2</td>
<td>93 (45.4%)</td>
<td>35 (37.6%) (31.0% - 44.3%)</td>
</tr>
<tr>
<td>3 or 4</td>
<td>65 (31.7%)</td>
<td>23 (35.4%) (28.8% - 41.9%)</td>
</tr>
<tr>
<td>Access to indoor avenue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>88 (42.9%)</td>
<td>29 (32.9%) (26.5% - 39.4%)</td>
</tr>
<tr>
<td>No</td>
<td>117 (57.1%)</td>
<td>47 (40.2%) (33.5% - 46.9%)</td>
</tr>
<tr>
<td>Access to outdoor courtyard</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>127 (61.9%)</td>
<td>49 (38.6%) (31.9% - 45.3%)</td>
</tr>
<tr>
<td>No</td>
<td>78 (38.1%)</td>
<td>27 (34.6%) (28.1% - 41.1%)</td>
</tr>
<tr>
<td>Subjective odour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>54 (26.3%)</td>
<td>13 (24.1%) (18.2% - 29.9%)</td>
</tr>
<tr>
<td>Woody</td>
<td>45 (21.9%)</td>
<td>16 (35.6%) (29.0% - 42.1%)</td>
</tr>
<tr>
<td>Weak “ammonia”</td>
<td>83 (40.5%)</td>
<td>39 (46.9%) (40.2% - 53.8%)</td>
</tr>
<tr>
<td>Strong “ammonia”</td>
<td>23 (11.2%)</td>
<td>8 (34.8%) (28.3% - 41.3%)</td>
</tr>
<tr>
<td>Avenue cleaning methods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swept or blower</td>
<td>108 (52.7%)</td>
<td>46 (42.6%) (35.8% - 49.4%)</td>
</tr>
<tr>
<td>Swept and watered down</td>
<td>97 (47.3%)</td>
<td>30 (30.9%) (24.6% - 37.3%)</td>
</tr>
</tbody>
</table>

3.1.4 Endoscopic Details
The numbers and percentages of horses with endoscopically visualised clinical signs related to the upper and lower respiratory tracts at Day 0 and over 2 weeks are shown in Table 3.4. The prevalence of neutrophilic IAD in horses with no visible tracheal discharge was almost half that of horses with visible discharge (15.5% vs 27.2%; P=0.01). In addition, the proportion of horses with neutrophilic IAD that had mucopurulent tracheal discharge was more than 2-fold higher than in horses with no discharge in the trachea (35.4% vs 15.6%; P=0.002) (Table 4).
When grade of PLH, tracheal discharge, and tracheal discharge quality increased in horses over 2 weeks, the prevalence of neutrophilic IAD in these horses was also higher than in horses in which grades remained the same or decreased. However, a lower proportion of airway inflammation in horses did not necessarily accompany a decrease in grade of these variables (Table 3.4).

Table 3.4: Number and percentage of horses with specific upper and lower respiratory tract endoscopic findings during examination on Day 0 and 14 and the proportion (with 95% CI) of horses that had neutrophilic IAD at Day 0 or developed neutrophilic IAD at Day 14.

<table>
<thead>
<tr>
<th>Variable and Categories</th>
<th>No. of horses (%)</th>
<th>No. with Neutrophilic IAD (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upper respiratory tract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLH grade (Day 0)*</td>
<td>n=235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>59 (25.1%)</td>
<td>12 (20.3%)</td>
<td>15.2% - 25.5%</td>
</tr>
<tr>
<td>2</td>
<td>151 (64.3%)</td>
<td>40 (26.5%)</td>
<td>20.9% - 32.1%</td>
</tr>
<tr>
<td>3</td>
<td>25 (10.6%)</td>
<td>4 (16.0%)</td>
<td>11.3% - 20.7%</td>
</tr>
<tr>
<td>PLH grade change</td>
<td>n=205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same</td>
<td>129 (62.9%)</td>
<td>49 (37.9%)</td>
<td>31.3% - 44.6%</td>
</tr>
<tr>
<td>Increase grade</td>
<td>30 (14.6%)</td>
<td>13 (43.3%)</td>
<td>36.6% - 50.1%</td>
</tr>
<tr>
<td>Decrease grade</td>
<td>46 (22.4%)</td>
<td>14 (30.4%)</td>
<td>24.1% - 36.7%</td>
</tr>
<tr>
<td><strong>Lower respiratory tract</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tracheal disch. (Day 0)</td>
<td>n=235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>97 (41.3%)</td>
<td>15 (15.5%)</td>
<td>10.8% - 20.1%</td>
</tr>
<tr>
<td>Int-cont blobs</td>
<td>81 (34.5%)</td>
<td>22 (27.2%)</td>
<td>21.5% - 32.9%</td>
</tr>
<tr>
<td>Uneven flecks</td>
<td>57 (24.3%)</td>
<td>19 (33.3%)</td>
<td>27.3% - 39.4%</td>
</tr>
<tr>
<td>Tracheal disch. change</td>
<td>n=205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same</td>
<td>108 (52.7%)</td>
<td>37 (34.3%)</td>
<td>27.8% - 40.8%</td>
</tr>
<tr>
<td>Increase grade</td>
<td>53 (25.9%)</td>
<td>23 (43.4%)</td>
<td>36.6% - 50.2%</td>
</tr>
<tr>
<td>Decrease grade</td>
<td>44 (21.5%)</td>
<td>16 (36.4%)</td>
<td>29.8% - 42.9%</td>
</tr>
<tr>
<td>Tracheal disch. Quality (TDQ; Day 0)</td>
<td>n=235</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>96 (40.9%)</td>
<td>15 (15.6%)</td>
<td>10.9% - 20.3%</td>
</tr>
<tr>
<td>Serous-seromucous</td>
<td>40 (17.0%)</td>
<td>6 (15.0%)</td>
<td>10.4% - 19.6%</td>
</tr>
<tr>
<td>Muco-purulent</td>
<td>99 (42.1%)</td>
<td>35 (35.4%)</td>
<td>29.2% - 41.5%</td>
</tr>
<tr>
<td>TDQ change</td>
<td>n=205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same</td>
<td>102 (49.8%)</td>
<td>34 (33.3%)</td>
<td>26.9% - 39.8%</td>
</tr>
<tr>
<td>Increase grade</td>
<td>57 (27.8%)</td>
<td>26 (45.6%)</td>
<td>38.8% - 52.4%</td>
</tr>
<tr>
<td>Decrease grade</td>
<td>46 (22.4%)</td>
<td>16 (34.8%)</td>
<td>28.3% - 41.3%</td>
</tr>
</tbody>
</table>

3.1.5 Serological and Bacteriological Results

Less than 5% of all horses seroconverted to any of the common respiratory viruses analysed (Table 3.5). However, the majority (>95%) of horses had previously been exposed to EHV1 and EHV4, or had recently been infected with ERV-A, and ERV-B, while approximately 76% of horses had not previously been exposed to Adenovirus. No horse seroconverted or had been exposed to Influenza virus. Approximately the same proportion of horses that developed neutrophilic IAD as did not had been exposed to these viruses (Table 3.5).

Only 8.3% of horses maintained an existing infection from Day 0 or developed significant bacterial numbers within their tracheas over 2 weeks in stables. However, 58.8% of these horses developed neutrophilic IAD, almost twice the proportion of horses that had no significant tracheal bacterial numbers (58.8% vs 35.1%; P=0.05) (Table 3.5).
Table 3.5: Numbers and percentage of horses and prevalence and 95% CI of neutrophilic IAD on Day 14, categorised by serological and bacteriological results.

<table>
<thead>
<tr>
<th>Variable and Categories</th>
<th>No. of horses (%)</th>
<th>No. Neutrophilic IAD Day 14 (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serological results</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EHV1</strong></td>
<td>n=211</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seroconversion</td>
<td>10 (4.7%)</td>
<td>5 (50%)</td>
<td>43.1% - 56.9%</td>
</tr>
<tr>
<td>No previous infection</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Previous exposure</td>
<td>193 (91.4%)</td>
<td>75 (38.9%)</td>
<td>32.1% - 45.6%</td>
</tr>
<tr>
<td>Recent infection</td>
<td>8 (3.8%)</td>
<td>1 (12.5%)</td>
<td>7.9% - 17.1%</td>
</tr>
<tr>
<td><strong>EHV4</strong></td>
<td>n=205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seroconversion</td>
<td>5 (2.4%)</td>
<td>1 (20.0%)</td>
<td>14.5% - 25.5%</td>
</tr>
<tr>
<td>No previous infection</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Previous exposure</td>
<td>191 (93.2%)</td>
<td>71 (37.2%)</td>
<td>30.6% - 43.8%</td>
</tr>
<tr>
<td>Recent infection</td>
<td>9 (4.4%)</td>
<td>4 (44.4%)</td>
<td>37.6% - 51.3%</td>
</tr>
<tr>
<td><strong>ERV-A</strong></td>
<td>n=207</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seroconversion</td>
<td>6 (2.9%)</td>
<td>4 (66.7%)</td>
<td>60.2% - 73.1%</td>
</tr>
<tr>
<td>No previous infection</td>
<td>9 (4.4%)</td>
<td>3 (33.3%)</td>
<td>26.9% - 39.8%</td>
</tr>
<tr>
<td>Recent infection</td>
<td>192 (92.8%)</td>
<td>73 (38.0%)</td>
<td>31.4% - 44.6%</td>
</tr>
<tr>
<td><strong>ERV-B</strong></td>
<td>n=204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seroconversion</td>
<td>3 (1.5%)</td>
<td>3 (100%)</td>
<td></td>
</tr>
<tr>
<td>No previous infection</td>
<td>1 (0.5%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Recent infection</td>
<td>200 (98.0%)</td>
<td>76 (38.0%)</td>
<td>31.3% - 44.7%</td>
</tr>
<tr>
<td><strong>Adenovirus</strong></td>
<td>n=202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seroconversion</td>
<td>1 (0.5%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>No previous infection</td>
<td>153 (75.7%)</td>
<td>59 (38.6%)</td>
<td>31.9% - 45.3%</td>
</tr>
<tr>
<td>Previous exposure</td>
<td>44 (21.8%)</td>
<td>15 (34.1%)</td>
<td>27.6% - 40.6%</td>
</tr>
<tr>
<td>Recent infection</td>
<td>4 (1.9%)</td>
<td>2 (50.0%)</td>
<td>43.1% - 56.9%</td>
</tr>
<tr>
<td><strong>Influenza virus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seroconversion</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>No previous infection</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Previous exposure</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Recent infection</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Bacteriology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Signif. growth (Day 0)</strong></td>
<td>n=205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>194 (94.6%)</td>
<td>74 (38.1%)</td>
<td>31.5% - 44.8%</td>
</tr>
<tr>
<td>Yes</td>
<td>11 (5.4%)</td>
<td>3 (27.3%)</td>
<td>21.2% - 33.4%</td>
</tr>
<tr>
<td><strong>Signif. growth develop'</strong> (Day 0-14)</td>
<td>n=205</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil or cleared</td>
<td>188 (91.7%)</td>
<td>66 (35.1%)</td>
<td>28.6% - 41.6%</td>
</tr>
<tr>
<td>Maintenance or develop'</td>
<td>17 (8.3%)</td>
<td>10 (58.8%)</td>
<td>52.1% - 65.6%</td>
</tr>
</tbody>
</table>
3.2 Results of All Data

3.2.1 Part I. Cohort Study: Non-infectious stable and environmental risk factors for development of IAD.

Of the 235 horses that were recruited, and data collected, at day 0, 138 were free of airway inflammation, 86 had some form of airway inflammation (comprising significant proportions of neutrophils and/or eosinophils and/or mast cells and/or lymphocytes) and 11 horses were excluded due to inability to interpret TA sample cytology (inadequate cells on smears).

Therefore, in the first analysis, 138 horses were eligible for study. Of these 138 horses, 110 developed the outcome as defined in Chapter 2 (IAD or non-IAD) after 2 weeks under racetrack stable conditions (Day 14). A total of 28 horses (28/138 or 20%) were either lost to follow-up (19 [14%]) or developed an “alternate” outcome such as eosinophilic or mixed IAD (9 [7%]). In the second analysis, 235 minus the 11 excluded horses, or 224 horses were eligible for study.

3.2.1.1 Analysis 1 (n=138)

3.2.1.1.1 Individual Risk Factors

Horses exposed over 2 weeks to better-ventilated looseboxes were significantly less likely to develop IAD than horses housed in looseboxes with no windows (P = 0.02). This finding was reinforced by the strong association between development of IAD in horses housed in looseboxes in which concentrations of CO₂ or ammonia were greater than 551ppm (P = 0.006) and 2ppm (P = 0.02) respectively. Horses exposed to high winds (>15 km/hr/week) (P = 0.03) and high evaporation (>5.7 mm/week) (P = 0.0007) were respectively 2.86 (95% CI: 1.04-7.91) to 3.83 (95% CI: 1.73-8.49) times more likely to have IAD than horses exposed to lower values.

It is important to note that most factors or exposures related to previous history prior to arrival at racetrack stables (eg., previous respiratory problem, distance travelled to stable, previous housing), training (eg., time since last worked, training stage, progress and activity), bedding (type, quality, age and depth), feed (type and whether or not it was wet down or placed on or off ground), other loosebox variables (eg., cleaning method, use of fans, access to other ventilation sources) as well as trainer, stable area and viral serology were not significant risks for IAD (P > 0.25) in this population of horses during the study period.

3.2.1.1.2 Risk Factors Considered Together

No significant interactions between risk factors were present. Table 3.6 shows details of adjusted odds ratios with 95% CI’s and the β coefficients for each factor important in the final analysis.

The odds ratio due to exposure to the ventilation proxy variable any window open in box, after adjusting for proportion of hard feed supplied, average mm of evaporation/week, average hours of sunlight/week and average km of wind /week was 0.15 (95% CI: 0.05 to 0.48) (P = 0.001).
Table 3.6: Adjusted Odds ratios and 95% confidence intervals for all relevant variables.

<table>
<thead>
<tr>
<th>Variable and Category</th>
<th>$\beta$ (SE)</th>
<th>Adjusted OR (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Box windows</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None open</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Any open</td>
<td>-1.914 (0.5656)</td>
<td>0.15 (0.15-0.45)#</td>
</tr>
<tr>
<td>Open air box</td>
<td>-0.7654 (0.5866)</td>
<td>0.47 (0.15-1.47)</td>
</tr>
<tr>
<td><strong>Proportion of hard feed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;50% chaff</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Even to &gt;50% corn/oat</td>
<td>-0.7476 (0.4639)</td>
<td>0.47 (0.19-1.18)#</td>
</tr>
<tr>
<td><strong>Average duration of sun</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-med (≤8.9 mean hr/wk)</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Long (&gt;9 mean hr/wk)</td>
<td>0.6918 (0.5002)</td>
<td>1.99 (0.75-5.32)#</td>
</tr>
<tr>
<td><strong>Average wind speed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (≤14.9 mean km/hr/wk)</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Med-high (&gt;15 mean km/hr/wk)</td>
<td>0.6609 (0.6187)</td>
<td>1.94 (0.58-6.51)#</td>
</tr>
<tr>
<td><strong>Average evaporation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-med (≤5.7 mean mm/wk)</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>High (5.7-10.3 mean mm/wk)</td>
<td>1.3287 (0.4789)</td>
<td>3.78 (1.48-9.65)#</td>
</tr>
</tbody>
</table>

#, Adjusted for all other confounders.
* Reference group.

3.2.1.2 Analysis 2 (n=224)

3.2.1.2.1 Individual Risk Factors

Similar to the first analysis, horses exposed over 2 weeks to better-ventilated looseboxes were significantly less likely to develop IAD as defined than horses housed in looseboxes with no windows ($P = 0.02$). This finding was reinforced by the weak association ($P = 0.06$) between development of IAD in horses housed in looseboxes in which concentrations of CO$_2$ were greater than 551ppm. Horses exposed to high evaporation (>5.7 mm/week) were 2.45 (95%CI: 1.32-4.56) times more likely to have IAD than horses exposed to lower values ($P = 0.004$).

Also in agreement with analysis 1, most factors or exposures related to previous history prior to arrival at racetrack stables (eg., previous respiratory problem, distance travelled to stable, previous housing), training (eg., time since last worked, training stage, progress and activity), bedding (type, quality, age and depth), feed (type and whether or not it was wet down or placed on or off ground), other loosebox variables (eg., cleaning method, use of fans, access to other ventilation sources) as well as trainer, stable area and viral serology were not significant risks for IAD ($P > 0.25$) in this population of horses during the study period.

3.2.1.2.2 Risk Factors Considered Together

Again, in this analysis, no interaction between risk factors was found. Table 3.7 shows details of adjusted odds ratios with 95% CI’s and the $\beta$ coefficients for each factor important in the final analysis.

The odds ratio due to exposure to the ventilation proxy variable any window open in box, after adjusting for cytological status of trachea at Day 0, age, average mm of evaporation/week and bacterial grade was 0.26 (95% CI: 0.12 to 0.60) ($P = 0.004$).
Table 3.7: Adjusted Odds ratios and 95% confidence intervals for all relevant variables.

<table>
<thead>
<tr>
<th>Variable and Category</th>
<th>β (SE)</th>
<th>Adjusted OR (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Box windows</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None open</td>
<td>0</td>
<td>1.00*</td>
</tr>
<tr>
<td>Any open</td>
<td>-1.3330 (0.4195)</td>
<td>0.26 (0.12-0.60)#</td>
</tr>
<tr>
<td>Open air box</td>
<td>-0.6633 (0.4312)</td>
<td>0.52 (0.22-1.19)</td>
</tr>
<tr>
<td><strong>Day 0 airway status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-IAD</td>
<td>0</td>
<td>1.00*</td>
</tr>
<tr>
<td>IAD</td>
<td>-0.3096 (0.4125)</td>
<td>0.73 (0.33-1.65)#</td>
</tr>
<tr>
<td>Eosinophilic component</td>
<td>-0.7717 (0.5863)</td>
<td>0.46 (0.15-1.46)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-36 months (“2yo”)</td>
<td>0</td>
<td>1.00*</td>
</tr>
<tr>
<td>36-48 months (“3yo”)</td>
<td>-0.8534 (0.4010)</td>
<td>0.43 (0.19-0.94)#</td>
</tr>
<tr>
<td><strong>Average evaporation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-med (≤5.7 mean mm/wk)</td>
<td>0</td>
<td>1.00*</td>
</tr>
<tr>
<td>High (5.7-10.3 mean mm/wk)</td>
<td>1.1860 (0.3559)</td>
<td>3.27 (1.63-6.58)#</td>
</tr>
<tr>
<td><strong>Significant bacterial growth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil or cleared over 2 weeks</td>
<td>0</td>
<td>1.00*</td>
</tr>
<tr>
<td>Cont or develop over 2 weeks</td>
<td>1.2787 (0.6278)</td>
<td>3.59 (1.05-12.29)#</td>
</tr>
</tbody>
</table>

#, Adjusted for all other confounders.
* Reference group.
3.2.2 Part II. Case-Control Study: Effect of endotoxin and (1→3)-β-D-glucan on neutrophilic inflammation of the lower airways in young thoroughbred racehorses in Sydney.

During the 22-month period, samples of total and respirable dust were collected from 112 horses. Of these 54 (48%) had IAD as defined when examined on either day 14 or 28 whereas 58 horses (52%) did not have IAD. Table 3.8 shows descriptive statistics (mean, median, minimum and maximum) of percentage of neutrophils in horses, and the concentrations of endotoxin, (1→3)-β-D-glucan, NH3 and CO2 found in Sydney racetrack stables. Cases were identified throughout the study period although there was a clear trend for fewer cases to occur during winter and spring. Three stables located at stable area 1 contributed 41 cases and 43 controls, 2 at stable area 2 contributed 12 cases and 14 controls and 1 at stable area 3 contributed 1 case and 1 control. Horses tolerated the dust sampling procedure very well.

Table 3.8: Descriptive Statistics for horses in which particle samples were analysed.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>Std Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Neutrophils</td>
<td>112</td>
<td>24</td>
<td>16</td>
<td>22</td>
<td>1.00</td>
<td>91</td>
</tr>
<tr>
<td>Respirable endotoxin (RE) (ng/m³)</td>
<td>108</td>
<td>3.89</td>
<td>2.20</td>
<td>5.69</td>
<td>0.18</td>
<td>44.57</td>
</tr>
<tr>
<td>Respirable glucan (RG) (ng/m³)</td>
<td>83</td>
<td>51.64</td>
<td>35.48</td>
<td>51.43</td>
<td>2.40</td>
<td>381.87</td>
</tr>
<tr>
<td>Total endotoxin (TE) (ng/m³)</td>
<td>96</td>
<td>48.31</td>
<td>33.04</td>
<td>60.82</td>
<td>3.42</td>
<td>507.29</td>
</tr>
<tr>
<td>Total glucan (TG) (ng/m³)</td>
<td>85</td>
<td>299.80</td>
<td>149.20</td>
<td>359.60</td>
<td>21.40</td>
<td>1900.00</td>
</tr>
<tr>
<td>Carbon dioxide (ppm)</td>
<td>106</td>
<td>679.57</td>
<td>620.00</td>
<td>267.99</td>
<td>410.00</td>
<td>1840.00</td>
</tr>
<tr>
<td>Ammonia (ppm)</td>
<td>112</td>
<td>1.91</td>
<td>&lt;2.0</td>
<td>2.15</td>
<td>&lt;2.00</td>
<td>17.40</td>
</tr>
</tbody>
</table>

3.2.2.1 Risk Factors Considered Together

After allowing for location prior to arrival at racetrack stables, bacterial growth, training progress, type of hay offered and carbon dioxide, the percentage of neutrophils found in TA samples collected from young racehorses in training increases linearly on average by 6.0% when exposed to 1ng/m³ of respirable endotoxin inhaled in dust in the horse’s breathing zone. The qualitative relationship is shown in Figure 3.4.

Figure 3.4: Percentage of neutrophils in TA using fitted values (1-40 ng/m³) for respirable endotoxin in the model equation demonstrating an initial exponential then linear relationship.

In addition, when all risk factors were considered together, several important, although non-significant relationships, existed between percentage of neutrophils and these factors. The adjusted percentage of neutrophils estimated by this analysis is shown in Table 3.9.
Table 3.9: Unadjusted and adjusted mean % neutrophils for confounding variables in final multivariate model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Unadjusted Mean % Neutrophils*</th>
<th>Adjusted % Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Previous location</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Stud/pre-training</td>
<td>67</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>• Spell/pre-training</td>
<td>45</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td><strong>Bacterial growth</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &lt;10^3CFU</td>
<td>106</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>• &gt;10^3CFU</td>
<td>6</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td><strong>Training progress</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Below expected</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>• Met expectation</td>
<td>103</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>• Above expectation</td>
<td>4</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td><strong>Hay type fed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Straw (high dust)</td>
<td>12</td>
<td>21</td>
<td>17</td>
</tr>
<tr>
<td>• Lucerne (medium dust)</td>
<td>74</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>• Clover grass (low dust)</td>
<td>12</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td><strong>CO2 concentration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &lt;500ppm</td>
<td>13</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>• 500-749ppm</td>
<td>74</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td>• 750-999ppm</td>
<td>10</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>• ≥1000ppm</td>
<td>8</td>
<td>37</td>
<td>31</td>
</tr>
</tbody>
</table>

* Adjusted for respirable endotoxin and all other (confounding) variables.

3.2.3 Part III. Case-Control Study: Association between inhaled endotoxin and (1→3)-β-D-glucan and occurrence of IAD in young thoroughbred racehorses in Sydney.

3.2.3.1 Risk Factors Considered Together
Young Thoroughbred racehorses were significantly more likely to develop IAD if exposed to < 1.2 ng/m^3 and > 4.2 ng/m^3 of respirable dust endotoxin, after adjusting for the confounding effects of sex of horse, hay-type fed, mm of evaporation/week, method of cleaning outside boxes and coughing any time during TA procedure (P = 0.03). The adjusted odds ratio for this effect of respirable endotoxin was estimated to be 4.34 (95% CI: 1.18 to 15.96) and 3.92 (95% CI: 1.06 to 14.49) respectively. The adjusted odds ratios and 95% CI for all relevant variables are presented in Table 3.10.
Table 3.10: Adjusted odds ratios (OR) and 95% confidence intervals for all relevant variables.

<table>
<thead>
<tr>
<th>Variable and Category</th>
<th>β (SE)</th>
<th>Adjusted OR (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respirable endotoxin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RE1 (0.2 - 1.2 ng/m³)</td>
<td>1.4679(0.6643)</td>
<td>4.34(1.18-15.96)#</td>
</tr>
<tr>
<td>RE2 (1.2 - 2.2 ng/m³)</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>RE3 (2.2 - 4.2 ng/m³)</td>
<td>0.7127(0.6689)</td>
<td>2.04(0.55-7.57)</td>
</tr>
<tr>
<td>RE4 (4.2 - 44.6 ng/m³)</td>
<td>1.3658(0.6670)</td>
<td>3.92(1.06-14.49)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colt</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Gelding</td>
<td>-1.0677(0.7860)</td>
<td>0.34(0.07-1.14)^</td>
</tr>
<tr>
<td>Female</td>
<td>-1.2650(0.7109)</td>
<td>0.28(0.07-1.14)</td>
</tr>
<tr>
<td><strong>Haytype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Straw</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Lucerne</td>
<td>-1.0784(0.5944)</td>
<td>0.34(0.11-1.09)^</td>
</tr>
<tr>
<td>Clover</td>
<td>-2.6205(1.0316)</td>
<td>0.07(0.01-0.55)</td>
</tr>
<tr>
<td><strong>Avenue cleaning method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broom</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Water/broom</td>
<td>-1.4995(0.5611)</td>
<td>0.22(0.07-0.67)^</td>
</tr>
<tr>
<td><strong>Evaporation grade</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low: 1.9 – 5.7 (mean mm/week)</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>High: 5.7 – 10.3 (mean mm/week)</td>
<td>1.3989(0.6042)</td>
<td>4.05(1.24-13.24)^</td>
</tr>
<tr>
<td><strong>Coughing during procedure</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No cough</td>
<td>0</td>
<td>1.0*</td>
</tr>
<tr>
<td>Cough as entering trachea</td>
<td>-0.3458(0.5251)</td>
<td>0.71(0.25-1.98)^</td>
</tr>
<tr>
<td>Cough during aspiration</td>
<td>0.9841(0.7311)</td>
<td>2.68(0.64-11.21)</td>
</tr>
</tbody>
</table>

#, Adjusted for all other confounders; ^ Adjusted for confounders including RE.
* Reference category.
4. Discussion and Conclusions

4.1 Study Design Issues, Bias and Limitations

Few studies investigating neutrophilic IAD in young thoroughbred racehorses have been performed, particularly under Australian racing conditions. The studies described in this report provided the strongest evidence to date about the distribution and determinants of this important equine respiratory disorder. In addition, these studies demonstrated the feasibility and success of performing population-based research using thoroughbred racehorses housed at racetrack stables in Sydney. Although it is attractive to generalise and apply the results of these studies to other horse populations throughout Australia, it is essential to keep in mind the differences in climate, housing types and stable management practices elsewhere in Australia as they may significantly impact upon the epidemiology of IAD in these populations.

Key Conclusion
Results of this report may be generalisable to other 2 to 3 year old racehorses in Australia.

There were many advantages of using a concurrent cohort design for this investigation of inflammatory airway disease. The most important advantage was that a general population of young thoroughbred racehorses could be identified from a geographically well-defined area at the beginning of the study and several factors related to their housing in racetrack stables could then be investigated prospectively by following these horses through time until the outcome of interest (ie., airway inflammation as assessed by > 20% neutrophils in TA samples) developed. Therefore, this design gave strong evidence that particular exposure factors preceding development of new cases of IAD as they occurred may play an important causal role in the genesis of this airway inflammation in young racehorses. Additionally, this study design allowed much inherent selection, measurement and confounding biases to be minimised. Most important was that the quality and extent of information collected for all horses regardless of whether they were exposed or non-exposed to particular factors was the same and therefore comparable, with nearly all measurements made using unbiased, objective or standard techniques. However, it is noteworthy that if error did occur in measurement of risk factors or during sample collection such error predictably decreases measures of effect or association making it more difficult to detect effects even if they truly exist. Therefore, any significant associations obtained in these studies are very likely to be genuine as this form of measurement error cannot be responsible for causing the observation of an association if one does not truly exist.

Key Conclusion
A well designed and executed cohort study gives very strong evidence for effects of risk factors on neutrophilic IAD.

In veterinary practice and research, collection of either a TA or BAL sample and analysing cytological findings from these fluid samples is the most common means of determining lower airway inflammation and IAD in horses. Whereas in the recent past, veterinarians used variable and unstandardised methods to collect TA and BAL samples, recently these methods were reviewed and a collection protocol agreed upon. In the studies reported here, TA samples were collected using only these latter standard techniques thereby maximising comparability between the latest studies and minimising error associated with non-standardised methods. Tracheal aspirate samples were collected using an endoscope to allow direct visualisation of the trachea and pooled saline, and quick and easy guidance of the catheter. In
addition, one experienced operator only, who was unaware of the horse identity, the day of collection and stable source, also evaluated smears of all TA samples.

Key Conclusion

Tracheal aspirate samples, collected using an endoscope and guarded catheter, were used to determine the presence of lower airway inflammation in racehorses because this sample is easily performed and interpreted, and widely accepted by racetrack personnel.

Implicit to accepting TA samples as a valid means of diagnosing lower airway inflammation in racehorses are a number of assumptions, which also potentially contribute to measurement error. One assumption is that interpretation of cytological analysis of samples, using differential counts of a particular inflammatory cell, can be based on specific “cut-off” percent values. In the studies reported here, great significance was attached to determination of lower airway inflammation using this cut off value, particularly for neutrophils, which, if above a certain percentage (20%), verified whether or not a horse had neutrophilic IAD. This measure consequently established which horses were cases and which were controls for the case-control study.

Although it is widely accepted that > 5% neutrophils in BAL samples indicates an inflammatory reaction within the lower respiratory tract, a corresponding cut-off value for the percentage of neutrophils in TA samples has not been universally accepted. The debate regarding the significance of high percentages of neutrophils within TA specimens is based on studies that found large variation in the percentage of neutrophils in TAs from apparently healthy horses and a poor correlation between results of TA and histopathology. As a result of this variation, the clinical usefulness of cytological evaluation of TA samples, using cut-off percent neutrophil values, for diagnosis of lower airway inflammation has been questioned, especially when applied to horses with chronic respiratory diseases. However, more recently, several studies have investigated TAs in young racehorses and demonstrated that in > 80% and > 73% of clinically normal racehorses neutrophils do not exceed 20% of the inflammatory cells present. Furthermore, Chapman et al (2000) reviewed 1235 TA samples from 724 horses in race training and found that in almost 90% of these horses neutrophil values were < 10%. In addition, Christley et al (2001) demonstrated a strong association between the presence of > 20% neutrophils in TA specimens and signs of respiratory disease (ie., coughing) in young racehorses. They concluded that greater than 20% neutrophils in TA samples from young racehorses was abnormal and indicated a significant inflammatory process and a strong risk of clinical respiratory disease. In view of these recent investigations a value of > 20% neutrophils in TAs were used in these studies as indicative of the presence of inflammation in young racehorses.

Key Conclusion
A cut-off of 20% neutrophils in TA samples has recently been accepted internationally as the reference point above which an abnormal inflammatory process in lower airways is diagnosed. Collection of TA samples and performing cytological analysis is the only definitive way of determining whether or not lower airway inflammation is present.

4.2 Descriptive Information

4.2.1 Losses to Follow-up
Losses of horses between Day 0 and Day 14 (13%) were acceptable and should not unduly bias estimates of effects and undermine validity of results of this study. Between Day 14 and Day 28, losses were higher as a result of greater numbers moving to alternate looseboxes or being moved from the stable complex for spelling. Horses were moved to alternate boxes by the trainer mainly for practical reasons, to accommodate new arrivals or to accommodate behavioural harmony between certain horses. The reason
that more 2-year-old horses were lost than 3-year-olds is probably related more to the need for younger horses to settle into a new environment rather than to their higher rate of wastage from injury, poor performance or respiratory disease, which is more likely further into their training regimen.  

4.2.2 Cumulative Incidence and Incidence Rate

The results of this study demonstrated that just over 40% of healthy two and three year old thoroughbred racehorses entering stables for training developed neutrophilic lower airway inflammation within the first 2 weeks of stay. Although prevalence can be simply defined as the number or proportion of affected animals, it is most meaningful when expressed as a proportion in relation to the number of animals in the population at risk of developing disease, not entirely possible in the current study. In dynamic populations such as the one used in this study in which horses periodically enter the study over time then “leave” if they develop the outcome (or when the study period [ie., 4 weeks] is completed) cumulative incidence was probably the best measure of the average risk of developing IAD during a particular period (the first 2 weeks of being housed in racetrack stables), in both the individual and the population. Also a proportion, cumulative incidence therefore was determined in preference to period or point prevalence calculations, although values obtained in this study for cumulative incidence were not inappropriately compared with prevalence figures given in other studies.

The cumulative incidence of 40% in the current study was similar to the prevalence of lower airway inflammation (33%) defined by Burrell et al (1996) found in a population of racehorses followed over time in a UK stable. However, a much lower prevalence was calculated (14%), (in which a similar method of calculation to the current study was used), in a longitudinal study of young thoroughbred racehorses in several training establishments. Differences in study design, in case (or IAD) definition, in population composition and age and calculation methods explain differences in measures of occurrence of IAD and reinforces the need for standardisation of definitions and measurements for IAD to allow comparability between studies.

Key Conclusion

The proportion of young thoroughbred racehorses that developed neutrophilic IAD (40.3%) within the first 2 weeks of entering racetrack stables for training is the first estimate of incidence of this condition in Australia. Most important it represents a very high percentage of affected horses that are clinically well and preparing for the high stresses of racing.

The results of this study also demonstrated that the incidence rate of lower airway inflammation was markedly higher than the rate calculated by Wood et al (1999). However, as with prevalence or cumulative incidence calculations, important design differences between these studies and the methods used for calculation of IR probably explain differences in values. In the current study, IR of IAD was calculated using horse-fortnight of observed data rather than a horse-month. Additionally, during the second fortnight the IR was more than 2 times lower than that determined during the first fortnight of risk (~20 cases per 100 horses per fortnight vs 56 per 100 per fortnight), much closer to values reported by Wood et al (1999) (8.9 cases per 100 horses per month or 4.5 per 100 horses per fortnight). Finally, underreporting of disease, due to less frequent measurements in the latter study compared to the current study, also served to explain discrepancy between incidence rates.

Another issue to consider with regard to the cumulative incidence of IAD was the variation in the extremes of incidence, given an ideal situation in which no losses of horses from the study occurred at any stage. If all horses that were lost within the first fortnight instead developed neutrophilic IAD (Figure 3.3) cumulative incidence of IAD in racehorses in the first 2 weeks of stabling might have been 9% higher than the estimated cumulative incidence of 40.3%. This represents a far higher proportion of horses that develop lower airway inflammation than previously reported and has important implications.
with regard to possible progression of inflammation to clinical disease, decreased training and racing performance and welfare issues.

**Key Conclusion**
*It is possible that at least 1 in 3 and at most 1 in 2 young thoroughbred racehorses that enter racetrack stables for training develop neutrophilic IAD within 2 weeks, and all are clinically healthy. However, in almost half of these racehorses lower airway inflammation persists and with increasing training and management stresses, may develop into clinical disease.*

### 4.2.3 Status of Horses on Arrival to Racetrack Stables

The results of this study showed that more than a third of horses entering racetrack stables for training had some form of airway inflammation (ie., neutrophilic [65%], eosinophilic [23%] or mixed [12%]). In addition, a higher proportion of these affected racehorses were 2 years old reflecting the greater susceptibility of 2-year-old racehorses compared to older horses to several types of health and performance-limiting disorders, which may ultimately result in their wastage to the racing industry \(^{122,335}\). Eosinophilic or mixed eosinophilic/neutrophilic IAD may result from migrating roundworm (Parascaris equorum) \(^{14,88}\), particularly in younger racehorses that might not be regularly dewormed at pre-training or spelling establishments prior to arrival at racetrack stables. Lower airway inflammation in which eosinophils predominate also may result from hypersensitivity reactions to inhaled allergens \(^{91}\). In addition, increased risk of neutrophilic airway inflammation and disease with decreasing age is well recognised and has been suggested to be associated with an immature immune response or less tolerance to specific aetiological agents \(^{122}\). Finally, a higher proportion of these affected horses were also colts although the reason for this is unclear.

This large proportion of affected horses entering racetrack stables for training represents an important subset of apparently clinically healthy horses that are placed under the stresses of training with IAD. In general, after 14 to 28 days housed in looseboxes, airway inflammation in approximately 50% of horses resolved but in many, some form of lower airway inflammation persisted. Although this study was unable to follow these horses for more than 4 weeks, under racetrack conditions, and increasing pressure from greater training demands, this lower airway inflammation, may progress to clinically overt disease. In addition, few to none of these horses had significant bacteria in airways, implicating exposure to non-infectious factors (in the case of neutrophilic IAD) at establishments prior to arrival as the agents likely responsible for the lower airway inflammation.

**Key Conclusion**
The conditions young racehorses are placed in and their health management (particularly with regard to regular anthelmintic treatment) prior to transport to racetrack stables may influence whether or not horses arrive with some form of IAD. However, the high proportion of young racehorses that develop IAD prior to arrival at racetrack stables are not necessarily at greater risk of continuation of inflammation or further respiratory disease.

### 4.2.4 Effects of Trainer and Racetrack Establishment

The results of the proportions of horses that arrived with or developed neutrophilic IAD within the first 2 weeks in stables for particular trainers and training establishments showed that there were important differences between particular trainers and training establishments. The significance of these effects is difficult to appreciate when considering basic descriptive statistics, and is dependent on assessment of all relevant risk factors together, as discussed in later sections.
4.2.5 Effects of Specific Management Details

The majority of trainers used sawdust bedding for looseboxes, with very few using straw or sand. The very low numbers of horses bedded on these latter two types of bedding reflects the declining popularity of these surfaces in Sydney racetrack stables, mainly as a consequence of difficulty with cleaning, maintaining depth and freshness and a perception they are more “dusty”. Although for most trainers there was no particular reason to place a specific horse in a box with straw or sand bedding, some trainers often placed only horses with musculoskeletal disorders in boxes with straw or sand. In addition, low numbers of horses housed on these bedding types probably precludes meaningful comparison between bedding types in later analyses.

In contrast to bedding, there was a clear trend for higher proportions of horses to have neutrophilic IAD if they were fed “high dust” hay (ie., straw-like), the proportion of horses with IAD decreasing when less dusty hay was fed (ie., clover). It has been shown that visibly dusty hay generates 10 fold more dust in looseboxes than good quality hay, which in turn yields approximately 10 times the respirable particles of processed or pelleted feeds 171. The higher exposure of horses to dust therefore may explain the higher proportion that developed IAD. In addition, the descriptive statistics for ventilation proxy variables demonstrated that ventilation is likely to be an important factor involved in horses developing neutrophilic IAD. This is discussed further in the next section.

Key Conclusion

Feeds that can potentially liberate high concentrations of dust into the air may also contain higher concentrations of “dust constituents” (such as pro-inflammatory agents and aerodallergens), and the challenge dose from these feeds will be even greater in the breathing zone of horses (ie., a hemisphere of 300 mm radius extending around the nostrils 214).

Finally, when outside sources of particles were cleaned using both broom and water, a lower proportion of horses developed IAD than when only a broom was used for cleaning. This suggests that, along with the evidence for improved ventilation and less dusty types of hay, decreasing exposure to dust (and the inflammogenic constituents of dust) by reducing overall airborne concentrations may play an important role in decreasing incidence of neutrophilic IAD.

4.2.6 Endoscopic Details

The descriptive results of endoscopic examination of the upper and lower respiratory tracts demonstrated a number of important features. When the degree of PLH worsened over the 2 weeks of study, the proportion of horses that developed IAD was higher, whereas when PLH grade decreased over 2 weeks, less horses developed IAD. This suggested that monitoring the change in PLH grade over time might be a useful practical indicator of developing lower airway inflammation for veterinarians who routinely perform endoscopy in young racehorses. In addition, worsening grade of PLH may also reflect ongoing challenge to the upper and lower respiratory tract defences and suggest increased or persistent exposure to inhaled infectious or non-infectious inflammogenic agents 354. However, it should be noted that the sensitivity of an increasing (or decreasing) grade of PLH to predict neutrophilic IAD likely would be relatively low due to the low proportions of horses that developed IAD overall.

Furthermore, when there was an increase over 2 weeks in the grade of tracheal discharge (eg., nil to intermittent or continuous blobs) or the character of the discharge (eg., nil to seromucous to mucopurulent), there was a trend for higher proportions of horses developing neutrophilic IAD. Similar to worsening PLH grade, these deteriorating tracheal findings may reflect persistent challenge by inhaled noxious particulate agents. However, it is important to note that similar proportions of horses with and without neutrophilic IAD, when examined at Day 0 (or at Day 14 or 28), had some form of tracheal discharge (flecks of intermittent to continuous blobs), emphasising the poor sensitivity of this endoscopic finding to detect neutrophilic IAD. This is in contrast to Wood et al (1999) who determined the sensitivity
of detecting IAD through observing moderate to greater amounts of mucus in the trachea as being 70.9% and the specificity as 90.3% \(^{11}\). However, it is difficult to compare these calculations between studies due to differences in design and in definitions for IAD and tracheal mucus grade. In addition, it is essential to keep these results in context because taken alone they may be misleading, and reveal little about the combined effects of all relevant variables.

**Key Conclusion**

*From a practical point of view, repetitive endoscopic examination may be useful to gauge the progression and nature of tracheal discharge and lend greater weight to the possibility of the presence of developing inflammation. However, single examinations and identification of minor discharge is unreliable as a mean to determine presence of neutrophilic inflammation.*

### 4.2.7 Serological and Bacteriological Details

The results of serology showed that young racehorse in this population rarely seroconverted to common respiratory viruses (herpes virus, adenovirus and rhinovirus). However, most racehorses had previously been exposed to EHV, most had recently been infected with ERV and few had previously or recently been infected with adenovirus. No horses seroconverted or were exposed to equine influenza virus. These results confirm the findings of several other studies \(^{4,8}\). In addition, approximately the same proportion of horses that developed neutrophilic IAD as did not had been exposed to these viruses, suggesting little relationship between viruses and IAD \(^{4,8}\).

**Key Conclusion**

*The evidence now overwhelming indicates that seroconversion to common respiratory viruses such as herpes virus, adenovirus and rhinovirus is not associated with development of neutrophilic inflammation in young racehorses entering racetrack stables. Most racehorses however, have recently or previously been exposed to these viruses and in the case of herpesvirus may remain in a latent state in horses.*

Furthermore, very few horses developed significant bacterial numbers in lower airways during the 2 weeks studied. However, in those horses that did develop infections over 2 weeks in stables, a majority (~60%) also developed neutrophilic IAD during the same period. Although it is impossible to know the exact pathophysiological events that occurred in airways of affected racehorses between Day 0 and Day 14 when samples were taken, it is possible that in this time frame and using this study design that significant numbers of bacteria found in airways preceded airway inflammation. In the small number of horses that developed significant bacterial numbers in lower airways, this may offer debatable evidence for a temporal relationship between bacterial colonisation and neutrophilic IAD.

**Key Conclusion**

*While there is a high likelihood that horses with mucopurulent or moderate to marked tracheal discharge have significant bacterial numbers in lower airways, \(^{4,11}\), bacteriological analysis of a TA sample is still the only definitive means of diagnosing a significant bacterial component to neutrophilic IAD, the type of bacteria and the most accurate antibacterial treatment.*

### 4.3 Overview of Analyses for Cohort and Case-Control Studies

#### 4.3.1 Non-infected stable and environmental risk factors for development of IAD.

The results of the first analyses of this study, in which only horses free of airway inflammation at the beginning (Day 0) were considered, demonstrated a number of significant associations between specific factors and development of neutrophilic IAD. It is important to note that these associations accounted for the confounding effects of other factors, emphasising the significance of the relationship.
Young thoroughbred racehorses were significantly less likely to develop IAD if housed in looseboxes in which ventilation was improved as a result of an open window(s) or if all four walls were open to constant airflow exchange over 2 weeks, than horses housed in looseboxes in which windows were absent. This result emphasises the importance of stable and individual box ventilation in preventing lower airway inflammation in racehorses, as has been suggested in many previous reports.  

**Key Conclusion**

*This study offers strong evidence that exposure to only 2 weeks of poor natural ventilation in looseboxes in which racehorses are housed is significantly likely to result in development of lower airway inflammation.*

In addition, this lower airway inflammation in horses was not accompanied by clinical signs of respiratory disease such as a cough or poor performance, a result also found in naïve horses housed in conventional stables with poor ventilation. Therefore, as stated in the previous section, a large proportion of young racehorses within 2 weeks of being placed in racetrack stables for training can have subclinical lower airway inflammation, which may or may not progress. The mechanism for this effect of poor ventilation is most likely due to the effects of increased concentrations of airborne dust and possibly noxious gases such as ammonia. Dust may contain sufficient concentrations of endotoxin to induce airway inflammation via direct proinflammatory effects on airway macrophages and other inflammatory cells.

It should be noted that the variable “windows open” was used as a proxy for ventilation quality. However, while proxy or surrogate variables for ventilation should be regarded as approximation of the true “ventilation”, these results indicate the relationship is very strong and should be an important consideration when designing looseboxes for young racehorses.

**Key Conclusion**

*It is clear that current stable design and reliance on natural ventilation are still inadequate to prevent development of lower airway inflammation in stabled horses. Increasing ventilation and reducing overall concentrations of particulate agents in looseboxes and stables could substantially prevent development of neutrophilic IAD and therefore decrease the likelihood of development of clinical respiratory disease.*

Our results also showed that after controlling for other factors, young racehorses were almost 3-4 times more likely to develop neutrophilic IAD if exposed on average to greater than 5.7 mm/2 weeks of evaporation than horses exposed to lower levels. The reason for this strong and unexpected effect of evaporation is unclear. Evaporation refers to emissions of water vapour from the surface of water at temperatures below boiling measured by calculating the millimetre reduction in a known volume of water. It is possible that high evaporative conditions may simply reflect levels of other meteorological factors such as high humidity, temperature and wind, all of which have some impact upon evaporation. High ambient temperatures and humidity are known to affect mucociliary function, which may adversely impair airway defences, while higher wind speed may increase airborne particle burdens within exposed stables. High temperatures combined with high humidity within stables, a common weather pattern found in metropolitan racetracks in Sydney, Australia, may also contribute to high release of spores and probably other particles from bedding such as straw, wood shavings and paper. Taken together, these environmental conditions probably increase airborne particulate burdens within looseboxes, which may increase insult to lower airway structures leading to neutrophilic inflammation. These conditions are therefore undesirable particularly in racehorses that are housed for long periods sometimes on dry dusty bedding (eg., poor quality straw) and often in poorly ventilated looseboxes at racetrack stables.
**Key Conclusion**

Exposure to persistently high environmental evaporative conditions is very strongly related to development of IAD in young thoroughbred racehorses. Although it is obviously impossible to control such conditions near and in stable establishments, awareness and anticipation of possible effects should prompt preventative measures to reduce the overall airborne dust burden in looseboxes (e.g., wetting down bedding and feed, use of fans to circulate air and draw particulate matter out of stables).

Similar results were demonstrated in the second analysis, in which all horses regardless of their airway status at Day 0 were considered. However, in this analysis, both age and development of significant bacterial growth within airways over 2 weeks also were significant risk factors for neutrophilic IAD. These findings contrast with the results of the previous analysis probably because a greater number of horses contributed information to the statistics.

As in the first analysis, there was a significant effect of an open window in looseboxes and lower evaporative conditions in decreasing the risk of IAD. The second analysis also showed that horses who developed over 2 weeks significant bacterial growth in airways, although only a very small proportion of the population (<10%), were almost four times more likely to develop neutrophilic IAD than horses with no bacterial growth in airways. Therefore, this could be interpreted to mean that the airways of those few horses that did develop bacterial infections of lower airways subsequently were infiltrated with high numbers of neutrophils (i.e., IAD) as a response to the challenge of overwhelming numbers of bacteria.

However, due to the 2 weekly collections of data, it is uncertain when in the 2 weeks significant numbers of bacterial growth occurred in relation to development of neutrophilic inflammation in airways. It is equally feasible that neutrophilic lower airway inflammation occurred as a consequence of continuous inhalation of high particle endotoxin concentrations (as discussed later). Attenuation in airway defences and altered airway surface (mucus and epithelium) during neutrophilic inflammation may result in the up-regulation of surface molecules, which may act as receptors for a wide spectrum of bacteria. Therefore, inflamed airways may be more susceptible to colonisation and infection.

**Key Conclusion**

Significant bacterial infection is rarely involved in acute subclinical neutrophilic IAD in young racehorses. However, in circumstances when they are present in significant numbers in airways, bacteria clearly play an important role in the perpetuation of IAD and if normal airway defences are impaired, this inflammation probably progresses to clinical respiratory disease, characterised by coughing.

The second analysis additionally showed that 3-year-old racehorses were significantly less likely to develop neutrophilic IAD than 2-year-old horses, a finding supported by several previous studies. As mentioned above, the proposed reasons for this age protective effect is related to a maturing immune response or greater tolerance to specific aetiological agents in older horses.

**Key Conclusion**

Realisation that 2-year-old racehorses (rather than older racehorses) are at a greater risk of lower airway inflammation should prompt trainers to carefully reconsider ways of avoiding challenges to the respiratory tract from airborne agents and to modify stable and perhaps training management practices to reduce “stresses” upon these young horses.

Finally, it is noteworthy that a sizeable number of risk factors that were analysed were not significantly associated with the development of neutrophilic IAD within the first 2 weeks of entering racetrack stables. These factors included abnormal tracheal health at Day 0, sex, trainer, stable area, factors related to current training history, bedding type, quality and age, other stable management factors such as avenue cleaning methods and use of fans, and exposure to common respiratory viruses.
Racehorses that arrived at racetrack stables with some form of IAD were not at greater risk of maintaining or developing neutrophilic IAD for 2 weeks when next evaluated. This is an important finding because it may suggest that persistence of individual horse and management factors, and exposures to noxious airborne agents is probably very important for maintaining IAD over short periods. However, recovery from IAD may also be the result of development of tolerance to inflammogenic risk factors. Sex was also not a risk factor for coughing in one study 122 but has not been investigated in other epidemiological studies investigating risks for IAD 8,11. Bedding type, quality and age over 2 weeks also was not associated with neutrophilic IAD, which may be a reflection of the high proportion of looseboxes (~90%) with bedding that was damp (usually wet down) and less likely to generate aerosolised noxious dust. Although ventilation proxy variables box window open and CO₂ grade were important risks, use of fans and cleaning methods outside looseboxes were not, and interactions between these variables were insignificant determinants of IAD. In addition, no significant association was found between seroconversion to common respiratory viruses and neutrophilic IAD, confirming the findings of other studies 4,8,11,161.

**Key Conclusion**

Contrary to expectation, many dust-generating activities and sources were not associated with IAD. However, in the case of bedding, probably too few horses were housed on straw and sand to give strong evidence for an effect.

### 4.3.2 The role of endotoxin and (1→3)-β-D-glucan in neutrophilic lower airway inflammation in young thoroughbred racehorses: A Case-Control Study.

In the first analysis of the case-control study, the effect of dust endotoxin and (1→3)-β-D-glucan, as well as several other factors, on the average percentage of neutrophils in lower airways of cases and controls were investigated. The results of this analysis demonstrated that only dust endotoxin and hay type were found to be significant risk factors for increases in neutrophil proportions in lower airways of young racehorses. The results showed that there was a significant linear (or exposure-response) relationship between the average percentage of neutrophils in TA and exposure to high respirable endotoxin concentrations in breathing zone dust. Figure 3.4 qualitatively shows that this relationship was initially exponential, but when exposure to endotoxin was above approximately 4.5 ng/m³ the relationship became linear implying that beyond a certain threshold, the higher the exposure to respirable endotoxin in inhaled organic dust, the greater the neutrophil accumulation into lower airways.

In contrast, the second analysis of the case control study was performed to compare the effect of specific risk factors, primarily endotoxin and (1→3)-β-D-glucan concentrations, between those racehorses that developed IAD (cases) and those that did not (controls). Similar to the results of the first analysis, this analysis demonstrated that young thoroughbred racehorses that developed IAD were approximately 4 times more likely to have been exposed to either low concentrations (<1.2 ng/m³) or high concentrations (>4.2 ng/m³) of dust endotoxin. Importantly, this association was highly significant while controlling the confounding effects of sex of horse, hay-type fed, millimetres of evaporation/2 weeks, method of cleaning outside boxes and coughing any time during TA procedure. The evidence therefore indicates strongly that there is a biphasic response to exposure to dust endotoxin in which horses exposed to low or very high concentrations are more likely to have neutrophilic IAD.

**Key Conclusion**

The reason for increases in proportions of neutrophils in lower airways as a result of exposure to dust endotoxin is most likely due to activation by endotoxin of macrophages and epithelial cells in airways.

Both macrophages and epithelial lining cells subsequently release pro-inflammatory mediators, which result in neutrophil invasion from airway walls and from circulation 239. This phenomenon is well recognised in acute inhalation 156,357, and observational studies 207,227,358,359, performed respectively in
healthy people and those exposed occupationally to organic dust. Furthermore, in human inhalation studies, the response of neutrophils in the airway to endotoxin occurs within 24 hours and if exposure persists, neutrophils can remain elevated in airways for approximately 10-14 days 239,241.

These results agree with experimental evidence found by Pirie and co-workers (2001) in horses, in which inhalation of high doses of LPS resulted in a dose response neutrophilia in lower airways (as measured using BAL) 128. However, the current study emphasises that inhalation of endotoxin at concentrations present in organic dust in many racetrack stables can result in increases in percentage of neutrophils in TA even though these concentrations can be well below “threshold doses” of respirable endotoxin used in inhalation studies. This is most likely because other agents present in stable dust such as moulds, glucans and proteases may enhance the inflammatory response to endotoxin 235. In addition, longer exposures in stable environments result in continuous and cumulative endotoxin challenge to horses 128.

**Key Conclusion**

*This study provides the first strong evidence that inhaled dust endotoxin is associated with and may cause neutrophilic IAD in young thoroughbred racehorses housed in racetrack stables. In addition, the higher the concentration of endotoxin inhaled by racehorses, the higher the proportions of neutrophils in lower airways. Although it is difficult to predict what dust sources contain higher endotoxin content, reducing overall airborne particle burdens in horse looseboxes and stables clearly is of paramount importance to health and welfare of racehorses.*

The reasons for both low and high concentration of dust endotoxin resulting in IAD are not clear. One explanation might be that the lower airway in horses exposed to mid-range concentrations of endotoxin developed an insignificant or a modified neutrophil response because the lower airway of some of these horses adapted to continuous or repeated low endotoxin exposures in inhaled dust 275. This cumulative low-dose exposure could have occurred in these horses given that all had been housed in variably dusty racetrack stables for at least 14 days prior to collection of TA and particle samples, and horse’s would have had some exposure to dusts in paddocks or yards before arrival at the racetrack stable. In contrast, it is possible that very small exposures to endotoxin resulted in higher neutrophil percentages in lower airways as a consequence of naivety in some horses (most were 2-year-olds). Subsequently, as tolerance to endotoxin develops 283, neutrophil proportions decrease although when exposure is high enough to overcome adaptive mechanisms or containing concentrations of other agents that interfere with defence mechanisms of the lower respiratory tract (the Cottesloe principle) 241, neutrophil percentages increase in an exposure-response manner. Tolerance to systemic endotoxin is a poorly understood, although well recognised phenomena in humans 280 and horses 289. The mechanisms responsible for down-regulation of macrophage activation as a result of repetitive LPS stimulation 280, while unclear, may involve alterations in LPS-dependent signal transduction pathways 281,282, production of LPS-neutralising compounds 283, or be a result of genetic factors 246,284.

**Key Conclusion**

*This study also found that individual racehorses respond differently to exposures to dust endotoxin. However, we suggest that the severity of neutrophilic IAD found on routine TA samples may be a useful marker of individual horse response to endotoxin, and which may prompt earlier intervention.*

Mould exposure is a major environmental factor implicated in a variety of respiratory disorders and in recent years, it has become increasingly clear that an important effect of moulds in humans is a non-specific airways inflammation as a result of the presence of (1→3)-β-D-glucans in their cell walls 147. The results of the case-control study demonstrate for the first time that dust glucan is present in stable environments frequently at very high concentrations. However, there was no relationship between inhalation of dust containing glucan and development of neutrophilic airway inflammation, after accounting for relevant confounding factors. Importantly there was no interaction between glucan and...
endotoxin or other relevant factors, suggesting that exposure to glucan likely plays only a minor role in
genesis of lower airway inflammation in young racehorses.

**Key Conclusion**

*This study indicates that exposure to (1→3)-β-D-glucan is not important, and by extension the lack of an
inflammatory effect probably implies that exposure to fungal agents also are unimportant in the genesis
of IAD.*

However, contrary to the findings of our analyses, exposure to fungal spores have been associated with
allergic IgE-related and non-specific neutrophilic inflammation in lower airways of horses\textsuperscript{134,143,144} and
humans\textsuperscript{145}. This inflammation may result directly from inhalation of fungi or from inhalation of volatile
chemicals produced by fungi, such as alcohols, aldehydes and ketones\textsuperscript{146}.

**4.4 Key Implications**

The implications of these findings could be far-reaching with regard to a greater understanding of the
sequence of events leading to lower airway inflammation and disease, and with respect to development of
management and therapeutic interventions to reduce exposure to dust and dust endotoxin. As with
humans, the severity of airway inflammation experienced by young racehorses exposed to organic dusts is
related to the endotoxin concentration of the inhaled dust. Therefore reducing airborne dust exposures and
possibly reducing endotoxin load within dust are critical steps in attempting to reduce the overall
incidence of this inflammatory disorder. This has important practical implications, specifically for stable
and ventilation design, and feed and bedding management. Horses housed in environments that encourage
high dust exposure may be more prone to the early developmental stages of IAD and therefore as training
progresses may be more likely to develop bacterial complications. Therefore, awareness and anticipation
of potential risk factors should prompt efforts to modify dust levels and training regimens, while
veterinarians could offer routine endoscopy and collection of TA samples, along with blood samples, as
part of the frequently performed “racing profile” work-up used by many trainers.
5. References


66


266. Michel O, Dentener MA, Corazza F, Buurman WA, Rylander R. Healthy subjects express differences in clinical responses to inhaled lipopolysaccharide that are related with inflammation and atopy. *Journal of Allergy and Clinical Immunology* 2001;107:797-804.


