Polymeric Airway Mucins in Equine Recurrent Airway Obstruction

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ABSTRACT

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ABSTRACT

In healthy airways, mucus forms part of the innate immune response protecting the respiratory epithelium from damage by pathogens and environmental debris (Rose and Voynow, 2006). Conversely, in many respiratory diseases, mucus becomes part of the airway disease pathology. Mucus hypersecretion along with reduced clearance can cause blockage of the small airways, impairing gas exchange, promoting inflammation and becoming a culture medium for bacterial colonisation (Thornton *et al.*, 2008).

Recurrent airway obstruction (RAO) is a common yet poorly understood equine chronic respiratory disease where such altered mucus properties and clearance have been identified as major factors in the disease pathology (Davis and Rush, 2002; Gerber *et al.*, 2000; Kaup *et al.*, 1990; Robinson, 2001). The gel-forming mucins are largely responsible for the transport properties of mucus. The major equine airway gel-forming mucin in health is Muc5b and to a lesser extent Muc5ac; produced in specialised respiratory epithelial goblet cells and sub-mucosal glands (Rousseau *et al.*, 2011b). Changes in mucin relative and net amounts and their macromolecular properties and interactions have been attributed to the altered physical properties of airway mucus in airways disease (Groneberg *et al.*, 2002a; Jefcoat *et al.*, 2001; Kirkham *et al.*, 2002; Robinson *et al.*, 2003; Sheehan *et al.*, 1995).

The project investigates the biochemical properties of mucins present in mucus from healthy horses and horses with RAO. This project identifies the anatomical presence of mucin-producing goblet cells and glands in fixed tissues from the respiratory tracts of healthy horses and subsequently examines mucin-production sites in respiratory tracts from horses with RAO. Finally the project investigates a methodology for the study of mucin production in airway cells harvested from live horses suffering from RAO.

Our investigations confirmed that horses with RAO have more endotracheal mucus than healthy controls, and that Muc5b is the predominant mucin with Muc5ac also present in RAO horse mucus, both during symptomatic disease and when horses are asymptomatic. Mucins are produced in epithelial goblet cells and sub-mucosal glands dispersed throughout the length and circumference of the equine trachea and bronchi. Goblet cell hyperplasia occurs in symptomatic exposed RAO horse airways, although goblet cells are smaller than in asymptomatic RAO horse airways. Exposure to a dusty stable environment is associated with more goblet cells per length of bronchial compared to tracheal epithelium in all horses. RAO horses have larger sub-mucosal glands containing more mucin than control horses.

Primary epithelial cell cultures grown at an air liquid interface are an alternative approach to study equine airway mucus, although the use of this culture system is in its early stages. We have developed novel ways to harvest equine airway epithelial cells (tracheal brushing) and shown it is possible to freeze cells collected via tracheal epithelial brushing in 20 % FBS and then culture to ALI at a later date.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

COPYRIGHT STATEMENT

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The pathway from equine clinician to laboratory researcher has not been an easy journey for me (or anyone trying to help me) during the course of my PhD. There have been bitter lows and rewarding highs; but ultimately I have learnt that persistence drives knowledge.

THE AUTHOR

Adele Williams is a veterinary surgeon (BVSc, 2003) and European specialist in equine internal medicine (DipECEIM, 2012). She graduated from The University of Bristol in 2003 and since then has worked to gain clinical specialisation in the field of equine internal medicine. During the path to specialisation she has undertaken clinical research projects in the areas of microbiology, clinical pathology and treatment of various clinical conditions. This PhD required a change of tack from clinical-based to more rigorous laboratory based research ethos.

ABBREVIATIONS

aa	Amino acid
Ab	Antibody
AB	Alcian blue
ALI	Air-liquid interface
ANOVA	Analysis of variance
AP	Alkaline phosphatase
BAL	Bronchoalveolar lavage
BCIP	5-bromo-4-chloroindol-3yl phosphate
BPE	Bovine pituitary extract
BSA	Bovine serum albumen
CF	Cystic fibrosis
cm	Centimetre
CK	Cysteine knot domain
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CsCl	Caesium chloride
cys	Cysteine-rich mucin region
Da	Daltons (measure of molecular mass)
DAB	3,3'-diaminobenzidine
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothrietol
FBS	Foetal bovine serum
GC	Goblet cell
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GuHCl	Guanidine hydrochloride
HCl	Hydrochloric acid
HRP	Horse radish peroxidase

IAA	Iodoacetamide
IAD	Inflammatory airway disease
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG-B	Biotin conjugated IgG
IL-4	Interleukin-4
IL-4R	Interleukin-4 receptor
IL-9	Interleukin-9
IL-13	Interleukin-13
IMS	Industrial methylated spirits
iu	International units
KDa	Kilo Daltons
M	Molar
MDa	Mega Daltons
MgCl	Magnesium chloride
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MS/MS	Tandem mass spectrometry
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NBT	Nitro blue tetrazolium
ng	Nanogram
PAS	Periodic-acid Schiff
PBS	Phosphate buffered saline
PLEH	Philip Leverhulme Equine Hospital
RA	Retinoic acid
RI	Refractive index
RAO	Recurrent airway obstruction
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate

SEM	Standard error of the mean
SG	Sub-mucosal gland
SPARAO	Summer pasture associated recurrent airway obstruction
SSC	Sodium chloride sodium citrate
STP	Spin tissue processor
TAE	Tris acetate EDTA
TBST	Tris buffered saline Tween
Th-1	Type 1 T helper lymphocyte
Th-2	Type 2 T helper lymphocyte
TNFα	Tumour necrosis factor alpha
TNS	Trypsin neutralising solution
TW	Tracheal wash (lavage)
URB	Urea reduction buffer
UV	Ultraviolet
v/v	Volume/volume
VNTR	Variable number tandem repeat
V_s	Volume of stored mucosubstance
vWF	von Willebrand factor
w/v	Weight/volume
µm	Micrometre (micron)

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

This thesis explores the polymeric airway mucins in equine airways in the equine inflammatory asthma-like disease, recurrent airway obstruction (RAO). This introduction will review the structure of the airways, the mucus gel and its major constituent macromolecules, the mucins, along with the mucin-producing cells of the airways. Our clinicopathological knowledge of RAO, highlighting the involvement of the respiratory mucus gel in the disease pathology is reviewed and then information on the secreted airway mucins in human diseases such as asthma, where current knowledge is lacking for equids, will be given before discussing what is currently known regarding mucins in equine airways.

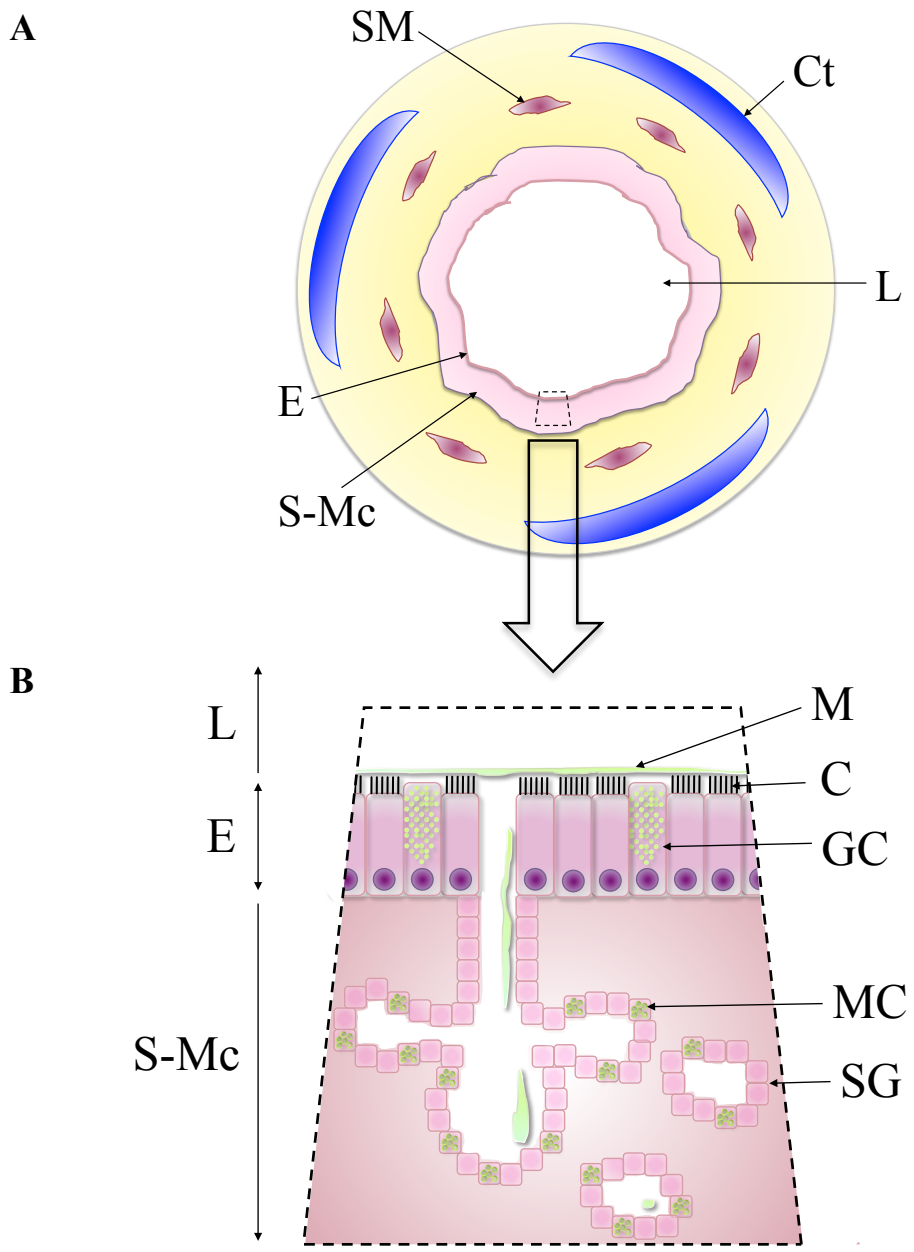
1.2 THE NORMAL AIRWAY

The airways function to deliver oxygen and remove carbon dioxide from the blood. Air reaches the lungs where gas exchange takes place with the blood, and is returned to the environment by a series of connected conducting airways. Horses are obligate nasal breathers thus air is inhaled via the nares, nasal cavity, through the pharynx and the larynx (limit of upper respiratory tract), down the trachea and into dividing progressively smaller tree-like branches of bronchi into the bronchioles and into the capillary-lined alveolar ducts and alveoli where gas exchange occurs; air is exhaled in the reverse direction (Robinson and Furlow, 2007). The trachea and bronchi are lined by a pseudostratified secretory epithelium with ciliated and non-ciliated cells above a basal layer of cells and sub-mucosal glands in connective tissue which is supported by cartilage and smooth muscle deep to the sub-mucosa (Pirie *et al.*, 1990a; Pirie *et al.*, 1990b; Robinson and Furlow, 2007) (*Fig. 1.1*). A layer of mucus lines the respiratory epithelium, keeping it moist and offering protection as part of the innate immune system.

The non-ciliated epithelial cells are predominantly secretory cells known as goblet cells that produce mucins, the molecules that provide the structural framework for mucus (Hovenberg *et al.*, 1996a; Thornton *et al.*, 1990; Thornton *et al.*, 2008). The sub-mucosal glands produce and secrete mucins and also serous secretions, which are released into the airway lumen and form the mucus gel (Hovenberg *et al.*, 1996a; Thornton *et al.*, 1990; Wickstrom *et al.*, 1998). The respiratory epithelium is covered by this mucus gel layer,

which interacts with the ciliated epithelium, termed the mucociliary escalator (Gerber and Robinson, 2007).

Fig. 1.1 **HEALTHY HORSE SMALL AIRWAY REPRESENTATION**. Schematic diagram representing **A**: a cross section of a generic healthy airway small bronchus with the airway lumen (L) lined by a layer of epithelium (E) over circumferential sub-mucosa (S-Mc) and supported by smooth muscle (SM) and cartilage (Ct); **B**: zoomed in diagram representing section of epithelium made up of ciliated (C) epithelial cells and non-ciliated mucin-secreting goblet cells (GC) covered by mucus layer (M); and sub-mucosa containing mucous cells (MC) in sub-mucosal glands (SG).



The bronchioles are not supported by cartilage and are the connection between the bronchi and the aleveloar ducts, they are lined by a single layer of cuboidal epithelium containing secretory Clara cells and fewer ciliated cells encircled by a layer of smooth muscle (Robinson and Furlow, 2007). Gas exchange occurs in the alveolar ducts and alveoli, which are single layer squamous and cuboidal epithelium in contact with pulmonary capillaries (Robinson and Furlow, 2007).

1.3 MUCUS

Mucus is a gel that covers non-keratinised luminal epithelial surfaces in the body, offering functions of protection, transport and lubrication. In the healthy respiratory tract, mucus as part of the innate immune response has a primarily protective role (Knowles and Boucher, 2002; Randell and Boucher, 2006). Mucus has antibacterial, anti-viral and even anti-fungal properties (Rogan *et al.*, 2006). Inhaled particles become entrapped within mucus and are then removed in the gel from the lungs by the mucociliary escalator.

The mucociliary escalator is formed by specialised ciliated epithelial cells lining the airways, whose cilia beat, continually moving the mucus layer away from the lung alveoli, clearing debris entrapped within the mucus to the pharynx where it is swallowed. In the horse, mucociliary clearance is faster when the horse's head is lowered, due to the effects of gravity and the anatomical positioning of the airways in this species (Gerber *et al.*, 1997; Racklyeft and Love, 1990). Early work to understand the function of mucus in the mucociliary escalator revealed that mucociliary transport function was lost in the absence of the mucus film, but could be restored with a variety of materials of the correct viscous and visco-elastic properties (King *et al.*, 1974; Meyer, 1976; Raidal *et al.*, 1996). Therefore a key property of respiratory tract mucus is that it is moveable and not static. The rate of mucociliary clearance in the horse is determined by the mucus gel physical properties, and the size and frequency of beating of the cilia lining the airways (Gerber *et al.*, 1997; Gerber and Robinson, 2007). The mean tracheal mucus velocity in healthy horses is has been measured to be between 1.5 - 2 cm/ min (Coombs and Webbon, 1987; Sweeney, 1989; Willoughby *et al.*, 1991).

The mucins or mucous glycoproteins are the major macromolecular components of mucus, providing its structural framework (Reid and Clamp, 1978; Thornton *et al.*, 2008). Sputum also contains water, other proteins and lipids, ions and deoxyribonucleic acid (DNA) (Creeth, 1978). Mucus is both elastic, with the ability to retain its original shape,

and viscous, being able to deform and flow. Mucins are believed to be responsible for the visco-elastic properties of mucus gels, but there is a lack of functional *in vivo* experimental evidence to support the actual protective role of mucins in health (Carlstedt *et al.*, 1985; Rogan *et al.*, 2006; Thornton *et al.*, 2008). What we do know is that in experimental knockout mice devoid of specific gastrointestinal mucins, the depleted barrier leads to chronic inflammation, they are unable to expel intestinal parasites, and are more likely to develop intestinal tumours (Hasnain *et al.*, 2011; Van der Sluis *et al.*, 2006; Velcich *et al.*, 2002).

Although in health mucus has a protective role, in diseases of the lower respiratory tract of man and horses, mucus accumulation is a common pathological feature (Robinson *et al.*, 2002; Rose and Voynow, 2006; Thornton *et al.*, 2008). The excess amounts of mucus that accumulate in the airways in respiratory disease may be due to mucus hypersecretion, reduced mucus clearance, or both (Thornton *et al.*, 2008). Investigation of airway mucus mucins in health and disease is important for understanding the potential changes that in these structural components of mucus that may occur leading to excess airway mucus in diseases such as RAO.

1.4 MUCINS

Mucins provide the structural framework for mucus; they prevent dehydration of the epithelial defensive gel barrier and present carbohydrate ligands to entrap pathogens (Felgentreff *et al.*, 2006; Kesimer *et al.*, 2009; Sheehan *et al.*, 2006; Thornton *et al.*, 2001; Van der Sluis *et al.*, 2006; Velcich *et al.*, 2002; Wickstrom *et al.*, 2000). Via binding with other secreted molecules from the respiratory epithelium, respiratory mucins provide a nidus for sequestration of host-protective proteins and peptides within the mucus gel (Felgentreff *et al.*, 2006; Thornton *et al.*, 2001; Wickstrom *et al.*, 2000). Mucins can be cell-membrane bound or secreted, of which there are polymer-forming and non-polymeric forms (Hattrup and Gendler, 2008; Thornton *et al.*, 2008). The secreted, polymeric, gel-forming mucins present in respiratory secretions are the focus of this thesis and we will discuss them further here.

The majority of current knowledge on respiratory mucins is a result of studies on human airway mucins; therefore the following sections will consider what is known about human secreted polymeric airway mucins and we will later summarise the limited current

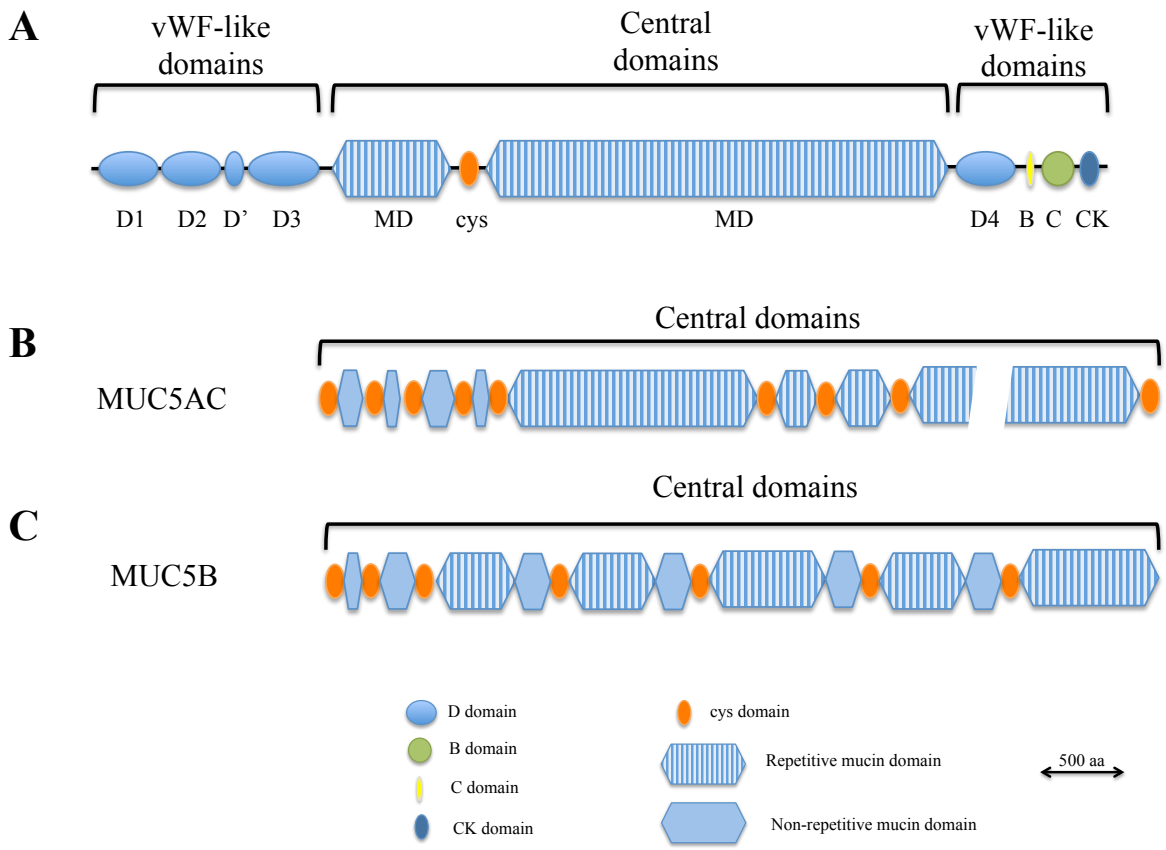
knowledge on equine respiratory mucins.

1.4.1 AIRWAY MUCIN GENES

There are 5 polymeric mucin genes, 4 of which have been shown to be expressed in human airways, namely *MUC2*, *MUC5AC*, *MUC5B* and *MUC19* (Buisine *et al.*, 1999; Chen *et al.*, 2004; Groneberg *et al.*, 2002b; Vinall *et al.*, 2000). In man, *MUC5AC* is expressed mainly in the airway epithelial goblet cells, *MUC5B* in the sub-mucosal gland mucous cells, *MUC2* at low levels in the bronchial epithelium and *MUC19* at low levels in the tracheal sub-mucosal glands (Buisine *et al.*, 1999; Chen *et al.*, 2004; Vinall *et al.*, 2000). *MUC2*, *MUC5AC* and *MUC5B*, all located on the same chromosomal gene complex, have highly conserved exon-intron sequences either side of their central domains (Pigny *et al.*, 1996; Rousseau *et al.*, 2004). Furthermore, all polymeric mucins have a similar gene structure, consisting of 5' and 3' regions flanking a large central exon (encoding the mucin domains), giving rise to a gene coding for a generic multi-domain protein model (*Fig. 1.2*) (Thornton *et al.*, 2008).

The highly conserved 5' and 3' flanking regions of *MUC5B*, *MUC5AC* and *MUC2* encode for cysteine-rich domains comparable with the von Willebrand factor (vWF) structural B, C, CK and D domains, which are important in glycoprotein polymerization via disulphide bonds (Buisine *et al.*, 1998; Desseyn *et al.*, 1997a; Desseyn *et al.*, 1998; Escande *et al.*, 2001; Rousseau *et al.*, 2004) (*Fig. 1.2*). The central large exon of the polymeric mucin genes encodes the mucin domains interspersed with a variable number of cysteine-rich regions (cys domains), which vary in length and repeats of sequences between the mucin species (Desseyn *et al.*, 1997b; Escande *et al.*, 2001; Rousseau *et al.*, 2004) (*Fig. 1.2*). The unique difference between the mucins lies in the mucin domain sequence and length, as coded for by the MUC genes, outlined for human MUC5B and MUC5AC in *Fig. 1.2* (Pigny *et al.*, 1996; Vinall *et al.*, 1998).

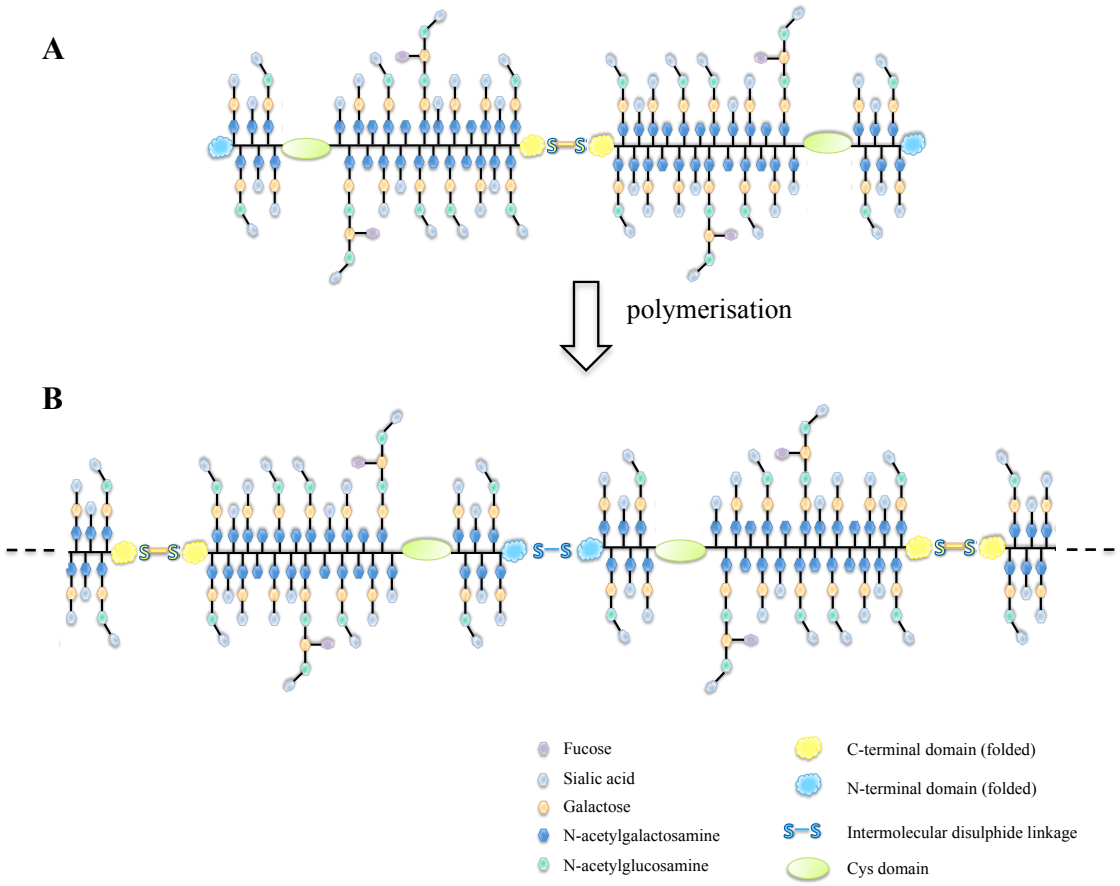
Fig. 1.2 **POLYMERIC MUCIN STRUCTURAL DOMAINS**. Schematic diagram representing human polymeric secreted mucin structural domains. **A**: Generic polymeric mucin polypeptide representation, with von Willebrand factor (vWF) like N- and C-terminal domains (B, C, CK and D domains), and a representation of a central region containing one cys domain and two mucin domains (MD). The central domain organisation for the human mucins **B:** MUC5AC and **C:** MUC5B are shown, demonstrating the repetitive and non-repetitive regions within the mucin domains. Figure adapted from Thornton *et. al*., (2008). aa denotes amino acid.



1.4.2 MUCIN MOLECULAR STRUCTURE

All mucins have a similar basic molecular structure. They are large complex O-linked glycoprotein polymers with a molecular mass of 10^6 to 50 x10^6 Da (Carlstedt *et al.*, 1983a; Clamp *et al.*, 1978; Reid and Clamp, 1978; Sheehan and Carlstedt, 1984b). Mucin polypeptides have at least one mucin domain, consisting of a large serine- and threonine-residue rich area (70 to 90 % of mucin weight) organised into densely packed clusters (Thornton *et al.*, 2008) (*Figs. 1.2, 1.3*).

Fig. 1.3 **MUCIN MOLECULAR STRUCTURE**. Schematic diagram representing generic polymeric secreted mucin structure (not to scale). Each mucin subunit is made up of highly glycosylated mucin domains interspersed with non-glycosylated cys domains (variable in number and insertion depending on the mucin). The mucin domains contain variable numbers of O-glycan chains, elongated with sugars such as N-acetylglucosamine (GlcNAc) galactose, fucose and sialic acid following addition of N-acetylgalactosamine (GalNAc) to serine and threonine residues. Mucin subunits dimerise via intermolecular disulphide linkages between C-terminal CK domains. Dimers are then polymerised via intermolecular disulphide bonds between their folded amino N-terminal D3 domains to form mucins of up to 50 MDa and 10 µm in length. Figure adapted from Thornton *et. al*., (2008). **A**: Generic mucin dimer. **B**: Representative section of a generic mucin polymer.



Studies of secreted polymeric airway mucins isolated from airway mucus have helped our understanding of the behavior of the molecules *in vitro*. These linear disulphide-linked polymers that form random coils in solution, are polydisperse in length (0.5 – 10 µm) and mass (2 – 50 MDa), due to the variable number of disulphide-linked subunits (Carlstedt *et*

al., 1983a; Davies *et al.*, 1996; Sheehan and Carlstedt, 1984a; Thornton *et al.*, 1990; Thornton *et al.*, 1991). Electron microscopy studies of human respiratory tract and cervical mucins have suggested that they possess linear, flexible thread-like structures (Lamblin *et al.*, 1979; Thornton *et al.*, 1990).

Mucins have a large range of O-glycans covalently attached via N-acetylgalactosamine (GalNAc) to the hydroxylated amino acids, serine and threonine. The mucin domains contain variable numbers of O-glycan chains, elongated with sugars such as N-acetylglucosamine (GlcNAc) galactose, fucose and sialic acid (*Fig. 1.3*) (Carlstedt *et al.*, 1983a; Thornton *et al.*, 2008). The large amount of sialic acid and sulphated sugar residues makes the mucins polyanionic and also extends their polypeptide size, leading to an expanded domain in solution. The mucin domains confer important structural and biological properties such as pathogen sequestration, water and ion binding and protease resistance on the mucins (Thornton *et al.*, 2008). The secreted polymeric mucins additionally have cysteine-rich (cys) domains interspersed between the highly glycosylated mucin domains, and at their amino (N) and carboxyl (C) termini, which enables them to form polymers via disulphide bonds, giving them their gel-forming ability (*Fig. 1.3*) (Perez-Vilar and Hill, 1999; Thornton *et al.*, 2000; Wickstrom and Carlstedt, 2001).

The above information regarding mucin macromolecular organisation has been gained from investigation of extracted and purified mucins solubilised in denaturing solvents and thus is unlikely to be representative of *in vivo* mucins, where complex reversible molecular interactions are likely to apply that will only be identified from the study of the non-native mucin form (Raynal *et al.*, 2003; Thornton *et al.*, 2008).

1.4.3 AIRWAY MUCINS: THE SECRETED GLYCOPROTEINS

In human airways the polymeric mucins MUC5AC and MUC5B have been identified as the determinants of the gel nature of respiratory mucus (Kirkham *et al.*, 2002; Sheehan *et al.*, 1999; Thornton *et al.*, 1996b). MUC2 has also been identified to a smaller degree (Hovenberg *et al.*, 1996b; Thornton *et al.*, 1996b). These secreted mucins all share the same macromolecular organisation as described above, in that they are disulphide bond-stabilised polymers which have variable numbers of disulphide-linked subunits making them polydisperse in mass and length (*Fig. 1.3*) (Davies *et al.*, 1996; Thornton *et al.*, 1990). Different glycosylated variants (low and high charge glycoforms) of MUC5B

have been identified (Thornton *et al.*, 1997).

As with most of our knowledge regarding mucins, studies on the synthesis and storage of the airway mucins has been largely focused on human airway mucin producing cells, which will be reviewed in the following section in order to consider what is needed to be studied in the horse.

1.4.4 SYNTHESIS AND STORAGE OF HUMAN AIRWAY MUCINS

Mucins are synthesised and stored, pre-formed in a compact, dehydrated state within granules in specialised secretory cells, namely goblet cells of the airway surface epithelium and in mucous cells of sub-mucosal glands (Hovenberg *et al.*, 1996a; Wickstrom *et al.*, 1998). MUC5AC and MUC5B are largely synthesised and stored in separate locations in human airways, namely the epithelial goblet calls for MUC5AC and the sub-mucosal glands for MUC5B (Hovenberg *et al.*, 1996a; Wickstrom *et al.*, 1998).

De novo synthesis takes 1 to 2 hours, yet cells are able to rapidly respond to particle inhalation challenge with increased mucus secretion due to storage of pre-formed dehydrated mucins within secretory granules (Sheehan *et al.*, 2004). Upon challenge at the airway surface (biological, physical or chemical insult), mucins are released and they hydrate, expand and rapidly form a protective network (Sheehan *et al.*, 2004). This can have pathological consequences, for example in asthma where in an acute attack, mucus hypersecretion occurs along with airway smooth muscle contraction causing obstruction of small airway which can ultimately be fatal (Sheehan *et al.*, 1995). Recurrent airway obstruction is a disease of horses similar to human asthma in which mucus hypersecretion is a key pathological component (Leclere *et al.*, 2011b).

In the following section, we will introduce and review our knowledge of the pathophysiology of the equine airway disease RAO, showing that excessive airway mucus is part of the disease pathology, before examining what is known about mucin alterations in human airway disease and then reviewing what we currently know about equine airway mucins.

1.5 EQUINE RECURRENT AIRWAY OBSTRUCTION

1.5.1 DEFINITION

RAO is the chronic recurrent condition of airway obstruction in mature horses, reversible by change of environment and or the use of bronchodilators (Robinson 2001). RAO is known in lay terms as "heaves", "broken wind" and "hay sickness", and is classically known to occur in stabled horses. An identical but less common clinical condition occurring in horses at pasture has been termed summer-pasture associated recurrent airway obstruction (SPARAO) (Mair, 1996; Seahorn and Beadle, 1993). Once affected, horses remain diseased for life; management and treatment can help reduce clinical symptoms, but there is no cure (Robinson, 2001).

The true prevalence of RAO in Great Britain has been estimated at around 14 % (95 % CI 10.7 to 17.45), based on a risk-screening questionnaire of geographically stratified horse owners in the UK (Hotchkiss *et al.*, 2007). The risk-screening questionnaire sampled a cross-section of horse breeds including Thoroughbred, Thoroughbred cross, Native pony, Arab, Warmblood and cross-breeds and found no breed-related risk factor for RAO (Hotchkiss *et al.*, 2007). Current estimates put the UK horse population at between 850,000 to 1,000,000 horses, equating to somewhere between 119,000 and 140,000 horses affected by RAO in the UK alone (Boden *et al.*, 2012; Robin *et al.*, 2013). Affected horses are frequently reported to suffer from exercise intolerance, and are unable to exercise during disease exacerbation (Dixon *et al.*, 1995b; Naylor *et al.*, 1992). RAO has a negative effect on performance potential, as suggested by exercise tolerance testing, thus limits the athletic capacity of affected individuals (Art *et al.*, 1999). Veterinary investigation, treatment and management of the disease undoubtedly have financial implications for horse owners, especially as the condition is life-long once it has developed. Thus the disease is important not only in terms of equine health and welfare, but also economic costs to individual horse owners and sports horse industries alike when the animals cannot be put to their normal use, whether that be as an elite athlete or ridden for pleasure.

RAO has historically been referred to as chronic obstructive pulmonary disease (COPD), and has been confused with the condition inflammatory airway disease (IAD). COPD is a chronic, progressive, irreversible human airway condition associated with smoking and so is now considered an inappropriate term for the reversible equine disease which more closely resembles human asthma (Dixon *et al.*, 2002; Robinson, 2001). In

fact, horses with naturally occurring RAO have been identified as a useful scientific model for human asthma (Leclere *et al.*, 2011b). Inflammatory airway disease is a rather vague term encompassing a gamut of equine lower airway diseases, but consensus is now generally accepted that it refers to the syndrome of reversible non-septic inflammation with mucus accumulation and associated reduced performance in young racehorses (Dixon *et al.*, 2002). The relationship between the syndrome IAD and RAO is unclear at present.

1.5.2 AETIOLOGY OF RAO

The precise aetiopathology of RAO is unknown and it is likely to be caused by a number of aetiological agents. Like asthma, there is a well-established association between both RAO and SPARAO and environment (Léguillette, 2003; Mair, 1996; Robinson *et al.*, 2001). It is widely thought that symptoms of RAO result from a specific hypersensitivity of a susceptible horse to an inhaled environmental allergen, such as fungal spores (Lavoie, 2007). However there is also evidence of a non-specific inflammatory response to multiple inhaled pro-inflammatory mediators such as stable dust (Lavoie, 2007). When RAO horses are housed in an environment in which they are exposed to their "trigger factor", whatever it may be (such as dusty hay), they develop clinical signs, becoming symptomatic. When the "trigger factor" is removed, for example moving the horse outside to an open field, the clinical signs subside and the horses become asymptomatic; however clinical signs reappear if RAO affected horses are reintroduced to their "trigger factor" (McGorum *et al.*, 1993a; McGorum *et al.*, 1993b). Experimentally, clinical signs have been induced in susceptible horses with isolated specific single allergens for example endotoxin or fungal spores and moulds such as *Faenia rectivirgula* and *Aspergillus fumigatus*; but many experimental models implement "natural challenge", feeding dusty hay and bedding on straw to induce clinical signs reliably and more effectively than purified single allergens (Derksen *et al.*, 1988; Fairbairn *et al.*, 1993; McGorum *et al.*, 1993a; McGorum *et al.*, 1993b; McPherson *et al.*, 1979; Pirie *et al.*, 2001).

The clinical signs of RAO become apparent in mature horses, and it is rarely diagnosed in horses less than 5 years of age. Two epidemiological investigations, one of over 1,400 horses in the USA, and one of over 800 horse owners in the UK, have identified increasing age as a risk factor for development of RAO. This has been confirmed with case series publications from various authors identifying mean age of horses presented for disease investigation to be from over 5 to over 12 years of age (Dixon *et al.*, 1995b; McPherson *et al.*, 1979; Naylor *et al.*, 1992; Vrins *et al.*, 1991). We do not currently know why younger

horses do not show clinical signs of RAO, it may be that the disease takes time to develop with immunological maturity before clinical signs become apparent.

There is undoubtedly a genetic heritable element to RAO, as there is with human asthma, with genetic and environmental factors influencing the course of disease (Holloway *et al.*, 1999; Ramseyer *et al.*, 2007). Several studies identify a genetic element of RAO with a complex basis of inheritance (Gerber *et al.*, 2009a; Jost *et al.*, 2007; Racine *et al.*, 2011; Swinburne *et al.*, 2009). RAO is most likely to occur in the offspring if both parents are affected, and more likely to occur if one parent is affected but can still occur if neither parent is affected with the disease (Marti *et al.*, 1991). Studies following the inheritance of RAO through two non-related Warmblood sires and their offspring have identified heterogeneity for the associated modes of genetic transmission, with one family having an autosomal recessive mode of transmission associated with equine chromosome 13, and the other having an autosomal dominant inheritance associated with equine chromosome 15 (Gerber *et al.*, 2009a; Jost *et al.*, 2007; Swinburne *et al.*, 2009). Furthermore, ingenuity pathway analysis of proteomic and genomic data from these families has identified the most common association to be based around the interleukin-4 receptor (*IL-4R*) gene (Racine *et al.*, 2011).

1.5.3 PATHOPHYSIOLOGY

On exposure by inhalation, RAO-affected horses within hours develop bronchoconstriction, airway inflammation and increased mucus secretion (summarised in *Fig. 1.4*) (Lavoie, 2007). These factors combined create small airway obstruction and resultant ventilation-perfusion mismatch, ventilation dead-space and hypoxaemia (Nyman *et al.*, 1991; Robinson, 2001; Robinson *et al.*, 1996). Chronically exposed affected horses develop airway remodelling with hypertrophied airway smooth muscle, epithelial goblet cell metaplasia, and fibrosis with collagen deposition (*Figs. 1.5, 1.6*) (Herszberg *et al.*, 2006; Leclere *et al.*, 2011a; Lugo *et al.*, 2006; Robinson *et al.*, 1996). The airway smooth muscle hyperplasia has been demonstrated to be reversible if horses are kept free from a trigger environment for a period of at least 12 months or 6 months if simultaneously treated with inhaled corticosteroids (Leclere *et al.*, 2012). The collagen deposition and fibrosis are also almost completely reversible with antigen removal and corticosteroid treatment (Lavoie, University of Montreal, Personal Communication).

Fig. 1.4 **RAO AETIOPATHOGENESIS: FACTORS ASSOCIATED WITH ONSET OF SYMPTOMS, PATHOPHYSIOLOGY AND ASSOCIATED CLINICAL SIGNS**. Recurrent airway obstruction (RAO) is a reversible inflammatory airway condition recognised in mature horses over 5 years of age. Affected horses become symptomatic, showing clinical signs when exposed to environmental trigger factors (lilac boxes). Reversible pathophysiological changes (blue boxes) can occur within hours (*) and also develop (^) with chronic exposure to trigger factors, combining to cause obstructions of the small airways, limiting gas exchange and air flow in the lungs, with the resultant clinical signs becoming apparent (green boxes). The degree of airway obstruction and severity of clinical signs is variable between individuals and dependent on duration of disease and extent of exposure. Removal of affected horses from trigger environment leads to reversal of pathophysiological changes, reduction of clinical signs and horses become asymptomatic; however clinical signs resume on re-exposure to the trigger environment.

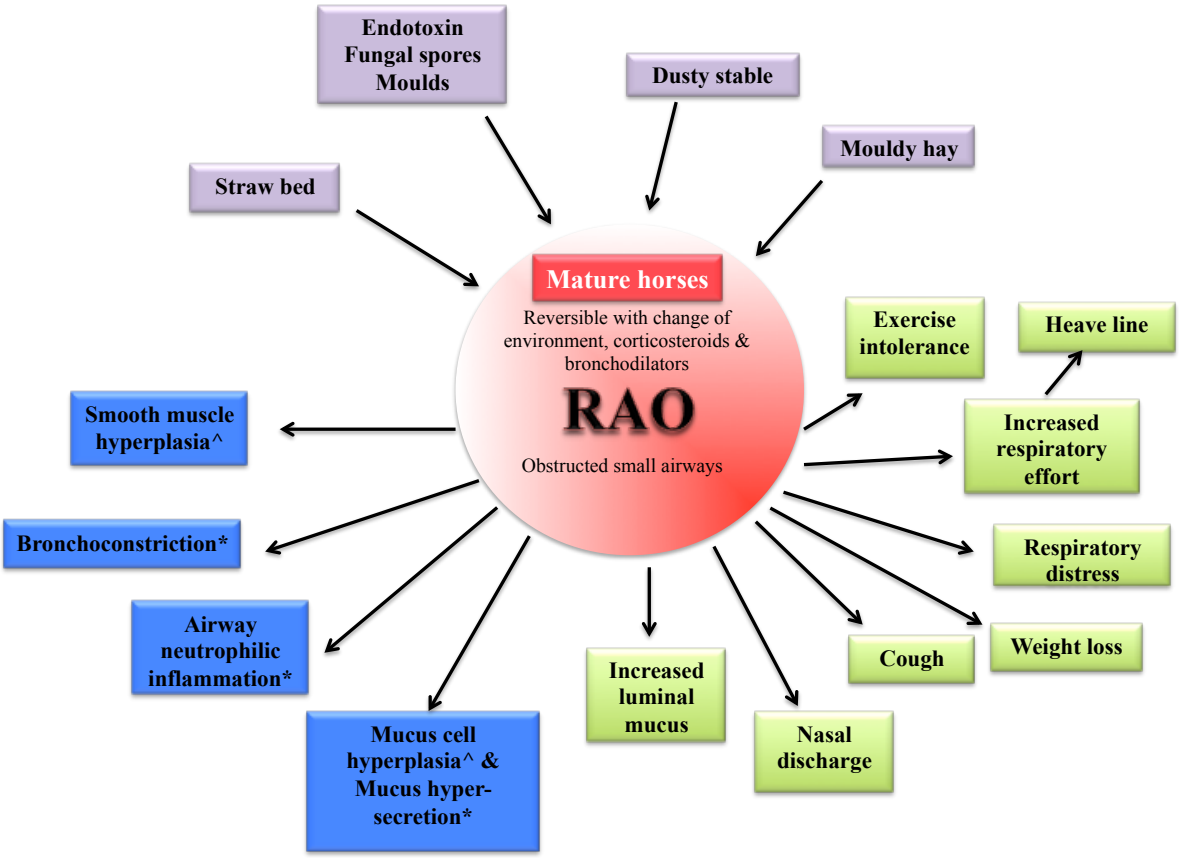


Fig. 1.5 **SYMPTOMATIC RAO HORSE SMALL AIRWAYS REPRESENTATION**. Schematic diagram representing **A**: a cross section of a symptomatic RAO horse airway small bronchus with broncho-constriction (BC) of the airway lumen (L) containing mucus hypersecretion (M HS) and neutrophilia (N); lined circumferentially by a layer of epithelium (E) over sub-mucosa (S-Mc) and surrounded by smooth muscle hyperplasia (SM H) and cartilage; **B**: zoomed in diagram representing section of epithelium demonstrating goblet cell hyperplasia (GC H), airway mucus hypersecretion and neutrophilia; and mucous cell hyperplasia (MC H) in sub-mucosal glands. (Refer to *Fig. 1.1* for schematic diagram of healthy small airways).

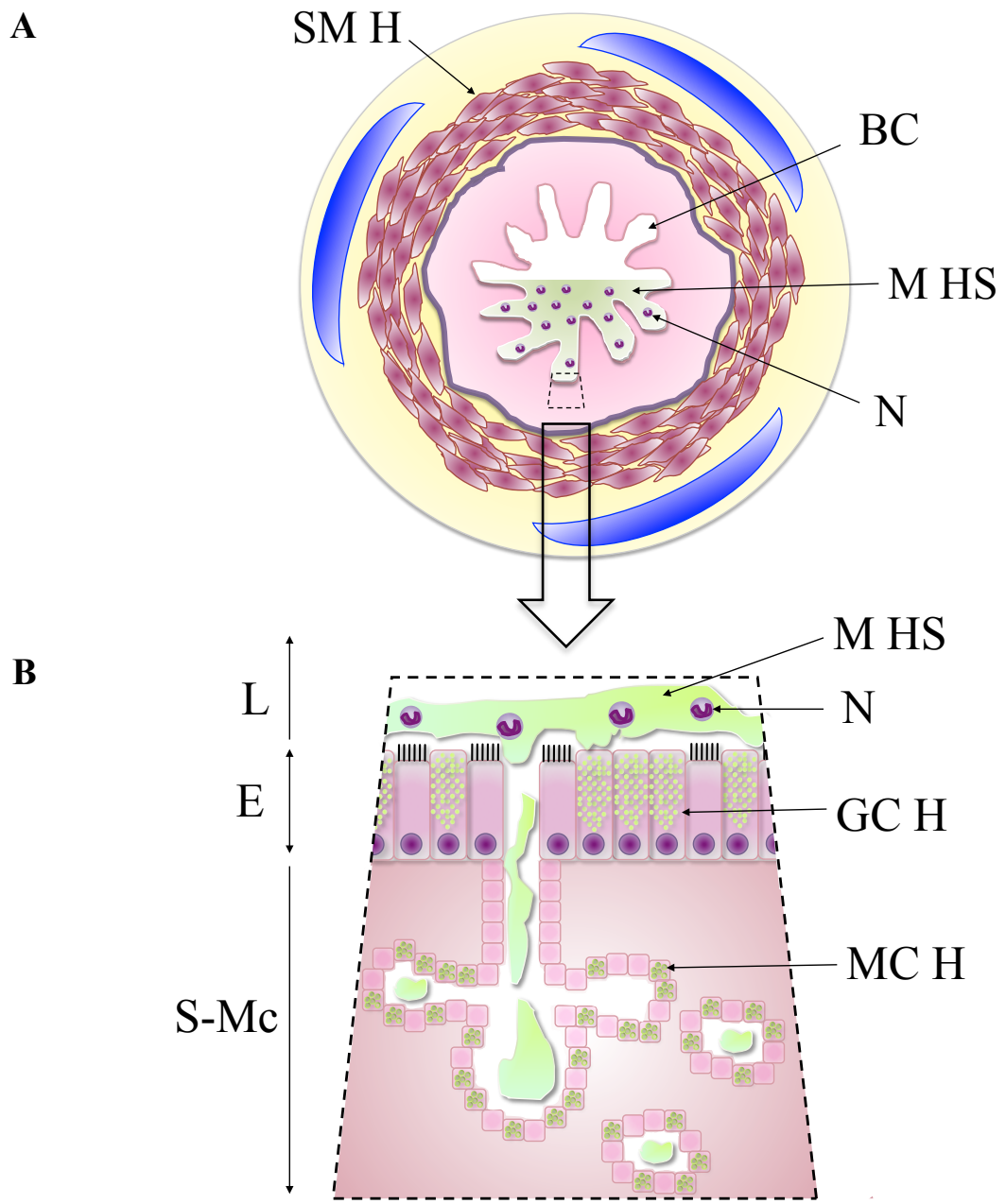
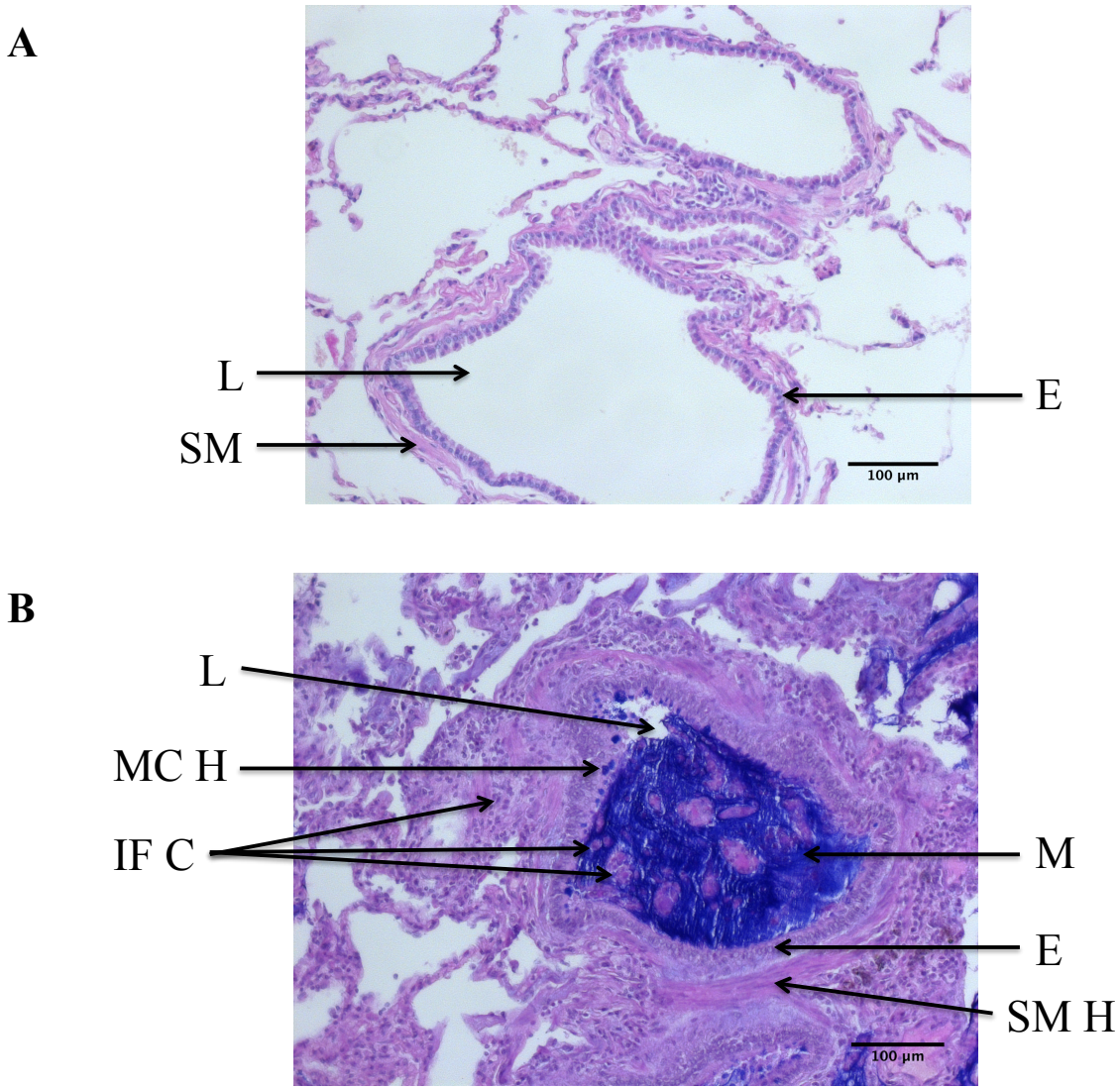


Fig. 1.6 **AIRWAY REMODELLING IN RAO**. Photomicrographs of cross-sections of equine bronchioles demonstrating healthy equine airways and histological small airway changes associated with RAO. **A**: Non-diseased horse airway showing small bronchiole lumen (L) lined by epithelium (E) and peri-bronchiolar smooth muscle (SM). No mucous cells are identifiable in the epithelium. **B**: RAO horse airway with peri-bronchiolar smooth muscle hypertrophy (SM H), mucus cell hyperplasia (MC H), luminal accumulation of mucus (M) and infiltration of inflammatory cells (IF C) into sub-mucosa and airway lumen. General mucin stain (periodic acid-Schiff's and alcian blue) with haematoxylin counterstain (x 20 original magnification; bar = 100 µm). (Images obtained from slides prepared for part of this PhD thesis).



RAO phenotypically resembles allergic disease classically induced by a Th-2 cytokine predominance. However, a neutrophilic, rather than eosinophilic, inflammation predominates the equine airways in RAO, with a rapid influx of neutrophils into the airways within 6 hours of environmental challenge (Fairbairn *et al.*, 1993; Pirie *et al.*, 2001). There is therefore controversy surrounding the immunologic basis of RAO. There is some evidence for similar pathophysiology in RAO to human atopic asthma, with IgE and Th-2 lymphocyte release of pro-inflammatory mediators (IL-4, IL-13) leading to airway smooth muscle contraction, vasodilation, vascular leakage and mucus hypersecretion; acting together to cause airway obstruction (Bowles *et al.*, 2002; Cordeau *et al.*, 2004; Husain and Kumar, 2005; McGorum *et al.*, 1993a; Pirie *et al.*, 2001). However, polyclonal antibodies used in some of these studies may not have been very specific, especially if there were high levels of IgG in the samples, causing cross-reactions (Wagner, 2009). Other studies have identified a Th-1 type immunological response with cytokines responsible for recruiting neutrophils (Ainsworth *et al.*, 2003; Ainsworth *et al.*, 2007; Ainsworth *et al.*, 2006; Debrue *et al.*, 2005; Giguère *et al.*, 2002). This has similarities with the non-eosinophilic asthma phenotype in people, which is hypothesized to be triggered by inhalation of a variety of environmental factors including endotoxin, pollutants and noxious gases (Douwes *et al.*, 2002; Fairbairn *et al.*, 1993; Haldar and Pavord, 2007; McGorum *et al.*, 1993a; Pirie *et al.*, 2001; Tremblay *et al.*, 1993).

In RAO, mucus overproduction as well as bronchoconstriction impacts on airflow and is a major contributor to airway obstruction (Gerber *et al.*, 2004) (*Fig. 1.6*). In severely affected cases mucus plugs causing airway obstruction negate the actions of bronchodilating drugs (Kaup *et al.*, 1990). Mucus of asymptomatic RAO horses have similar rheological properties to mucus of healthy horses; however symptomatic RAO horses have increased viscoelasticity and decreased calculated mucociliary clearability and cough clearability of their tracheal mucus (Gerber *et al.*, 2000). Therefore an unfavourable change to the rheological properties of mucus of RAO horses may occur on exposure to a challenge environment, contributing to stasis and accumulation of mucus in clinically affected RAO horses.

1.5.4 CLINICAL SIGNS OF RAO

The reversible clinical signs of RAO can vary from occasional coughing and

mucopurulent nasal discharge, progressing to exercise intolerance, severe cough and respiratory distress (Léguillette, 2003; McGorum *et al.*, 1993b; Robinson *et al.*, 1996) (*Fig.1.4*). Symptoms become apparent in RAO horses each time they are exposed to a challenge or trigger environment; this is reversible if the horse is removed from the trigger environment, where the horse recovers becoming asymptomatic (Léguillette, 2003; McGorum *et al.*, 1993b; Robinson *et al.*, 1996). Clinical signs tend to worsen and progress if horses are continually exposed to their trigger environment (Léguillette, 2003; McGorum *et al.*, 1993b; Robinson *et al.*, 2001; Robinson *et al.*, 1996).

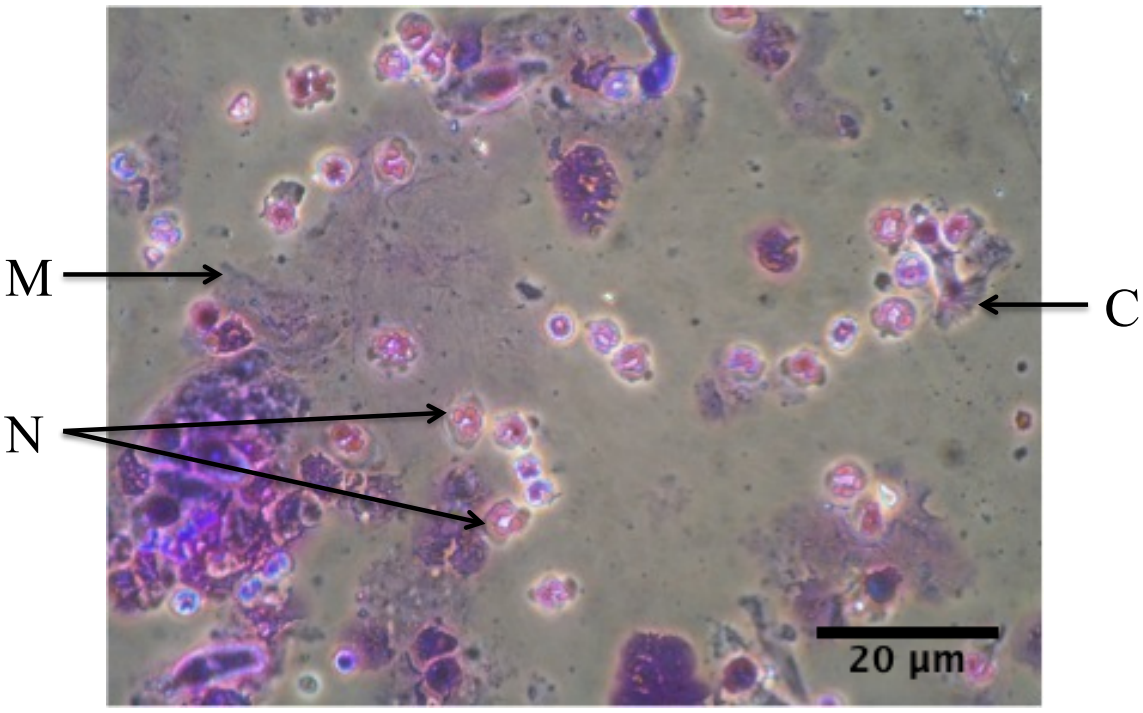
During RAO exacerbation, respiratory rhythm changes, with forced, prolonged expiration and extra abdominal effort; over time this leads to development of a well-defined "heave line", due to hypertrophy of the external abdominal oblique muscle (Couetil *et al.*, 2001; Léguillette, 2003; McPherson *et al.*, 1978). Severe exacerbations are accompanied by an increased respiratory rate with flaring of the nostrils, and horses may fail to gain weight or suffer weight loss from the energy drain required for breathing (Léguillette, 2003; Mazan *et al.*, 2004). RAO horses are often exercise intolerant, even when asymptomatic (Art *et al.*, 1999; Dixon *et al.*, 1995b; Naylor *et al.*, 1992).

1.5.5 DIAGNOSIS OF RAO

Clinically, a presumptive diagnosis of RAO is usually based on a combination of history, age, stabling conditions, and clinical examination findings (Lavoie, 2007). A typical history would be of a stabled mature horse (over 5 years of age) with a normal appetite and absence of pyrexia, presenting for persistent cough, increased respiratory effort, abdominal heave line and abnormal lung sounds on thoracic auscultation. Diagnosis is confirmed by demonstration of neutrophilia in airway secretion cytology (*Fig. 1.7*) and reversibility of the condition with environmental changes and bronchodilating drugs (Léguillette, 2003; Robinson *et al.*, 2001).

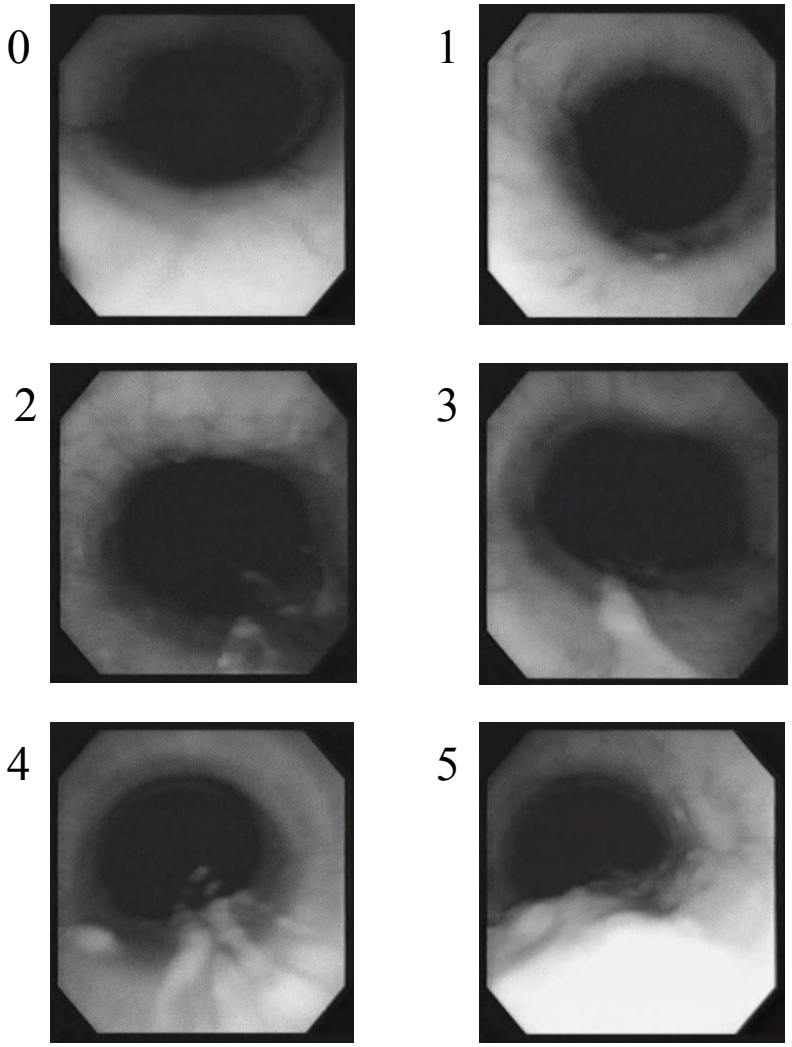
Tracheal endoscopy provides an indication of the degree of tracheal epithelial inflammation and allows direct visualisation of the amount and consistency of airway mucus with simultaneous tracheal mucus collection (tracheal lavage or wash, TW) and performance of bronchoalveolar lavage (BAL). TW and BAL are both techniques of collecting airway secretions via instillation and retrieval of sterile saline into the airways (Davis and Rush, 2002; Dixon *et al.*, 2002; Smith *et al.*, 2002).

Fig. 1.7 **PHOTOMICROGRAPH OF AIRWAY SECRETION CYTOLOGY FROM A HORSE SUFFERING FROM RAO**. Endoscopically guided infusion of sterile 0.9 % saline and subsequent collection via suction yields airway secretions for microscopic cytological examination. Typical findings in tracheal lavage sample from an RAO horse are shown, including neutrophils (N), background mucus (M) and sloughed cliliated epithelial cells (C). Haematoxylin & eosin stain (x 40 original magnification; bar = 20 µm). This slide was prepared from equine airway mucus collected as part of this PhD thesis.



Tracheal mucus accumulations are reported both during clinical exacerbations of RAO and in remission, and can be found in the absence of other clinical signs (Christley *et al.*, 2001; Jefcoat *et al.*, 2001; Robinson *et al.*, 2002; Robinson *et al.*, 2003). Mucus and tracheal inflammation scoring systems have been employed to provide more structure to subjective assessment (Dixon *et al.*, 1995a; Gerber *et al.*, 2002; Gerber *et al.*, 2004; Smith *et al.*, 2002). A widely used mucus score with a scale of 0 to 5, where 1 is a small volume or small blobs of mucus and 5 is extreme profuse amounts of mucus (*Fig. 1.8*) has been shown to have good inter-observer agreement for mucus accumulation score, proving a reproducible measure of mucus volume (Gerber *et al.*, 2004). Ultimately though these scores remain subjective, and the presence of mucus in the airways does not always correlate with airway inflammation and clinical signs (Christley *et al.*, 2001).

Fig. 1.8 **ENDOTRACHEAL MUCUS SCORING SYSTEM**. Visible mucus noted during video-endoscopy is graded on amount present in the trachea based on a published endoscopic tracheal mucus scoring system Gerber *et al*., (2005). 0: None, clean. 1: Little, multiple small blobs. 2: Moderate Larger blobs. 3: Marked, confluent, stream-forming. 4: Large, pool-forming. 5: Extreme, profuse. Images captured from video obtained during endoscopy of horses as part of this PhD thesis.



1.5.6 TREATMENT

The mainstay of treatment is removal from the environment in which the condition is exacerbated. Anti-inflammatory drugs, principally glucocorticoids, and bronchodilators are often used, at least initially in the acute stages of disease to help resolve clinical signs of respiratory distress (Durham, 2001; Leclere *et al.*, 2010; Léguillette, 2003). Inhalation therapy is superior in effect and advantageous for drug targeting whilst using lower doses

compared to other routes of administration (oral, intravenous, intramuscular) for steroids and bronchodilators (Allen and Franklin, 2007). Dembrexine, a mucolytic agent, is the only product licensed in the UK for treatment of increased respiratory mucus in horses. Dembrexine it is commonly sold in the UK but there is a lack of peer-reviewed evidence to support its use, the clinical field trials by the drug company not demonstrating a statistically significant difference with an untreated control group, and it is therefore of questionable efficacy at present (Lavoie, 2007; Matthews *et al.*, 1988). Cases of RAO can be difficult to manage, especially where it is impossible or impractical to remove the causal trigger factor (Léguillette, 2003).

The remainder of this introduction will consider what is currently known about airway mucins in disease, referring to knowledge gained from studies on human airway mucins to gain an insight into the possible mechanisms behind the excess airway mucus and potential mucin altered physical properties in horses suffering from RAO.

1.6 MUCINS IN HUMAN AIRWAY DISEASE

1.6.1 GENETIC ASSOCIATIONS OF MUCINS AND HUMAN AIRWAY DISEASE

Mucin domains encoded for in exons show genetic variation, and variable number tandem repeat (VNTR) polymorphisms have been shown to lead to differences in the gene product size for several mucin genes (Vinall *et al.*, 1998). Variations in the chromosomally linked polymeric airway mucin genes, *MUC5AC* and *MUC5B* and their allelic linkage with *MUC2*, which has been shown to have different VNTR alleles in asthmatic compared to normal people, are likely to be associated with occurrence of asthma in people (Rousseau *et al.*, 2007; Vinall *et al.*, 1998). The mucin domain size of the *MUC5B* and *MUC5AC* genes do not show large size variation however; thus functional variations affecting the translated glycoproteins may occur (Thornton *et al.*, 2008).

In the following section of this thesis we will consider the two main secreted gel-forming mucins found in human airways, MUC5AC and MUC5B and what we know about them in airway disease.

1.6.2 MUC5B AND MUC5AC IN HUMAN AIRWAY DISEASE

Overproduction of mucus and altered transport properties of mucus occurs in human airway diseases such as asthma, cystic fibrosis (CF) and COPD. The polymeric mucins that are the key structural components of the mucus gel, if changed in relative amounts and biochemical nature in disease, may also be key to the pathological properties of the mucus. Hence, various studies to understand the physical and biochemical properties of the human major airway gel forming mucins MUC5AC and MUC5B in human airway disease have been undertaken.

MUC5AC appears to be the predominant mucin in airways gel in healthy humans, with the ratio of MUC5B to MUC5AC changing in disease, favouring MUC5B (Kirkham *et al.*, 2002). In human asthma, CF and COPD patients, increases in the polymeric mucins MUC5B and MUC5AC have been found in expectorated sputum samples compared to normal human sputum (Kirkham *et al.*, 2002). This is in agreement with studies demonstrating goblet cell hyperplasia in asthmatic airways (Fahy, 2002). Indeed, MUC5B has been found to be the predominant mucin in both CF and COPD (Burgel *et al.*, 2007; Kirkham *et al.*, 2002). In addition to this, on analysis of the two MUC5B isoforms, a higher proportion of the low charge MUC5B glycoform was found in mucus from an asthmatic respiratory plug (Sheehan *et al.*, 1999).

Mucins collected from human patients with cystic fibrosis have been shown to have the same architecture and macromolecular properties as respiratory secretions from normal human samples (Thornton *et al.*, 1991). A study comparing macromolecular properties of mucins from healthy patients and patients with chronic bronchitis found the molecules to have the same basic macromolecular structure but differing charge densities, suggesting alterations in the mucin structure in disease (Davies *et al.*, 1996). Analysis of the low-charge glycoform of MUC5B isolated from a mucus plug of a patient that died in *status asthmaticus* had altered macromolecular architecture, being branched and cross-linked rather than linear on electron microscopy examination (Sheehan *et al.*, 1999). Mucin alterations noted in human airway disease highlighted here include increase in amount of mucin, relative ratios of MUC5AC to MUC5B and the MUC5B glycoform ratios in sputum samples, as well as one example of macromolecular structural changes.

As the clinicopathological findings and inflammatory pathways in RAO are most akin to human asthma, knowledge of mucin properties in asthma could prove insightful for

consideration of the role of mucins in RAO. Therefore we will discuss what is known about MUC5B and MUC5AC in asthma in the following section.

1.6.2.1 MUC5B AND MUC5AC IN ASTHMA

Gene expression of mucins in airway tissues from asthmatic patients has been evaluated. A marked increase in MUC5AC expression in epithelial samples obtained via bronchoscopic biopsy has been found in asthmatic patients (Ordonez *et al.*, 2001). Furthermore, amounts of MUC5AC stored glycoprotein were increased as measured by semi-quantitative assessment of immunohistochemical stain intensity of biopsied tissues from normal and asthmatic patients (Ordonez *et al.*, 2001). Induced sputum obtained from asthmatic and healthy individuals showed an increase in amounts of both MUC5B and MUC5AC in the asthmatic patients (Kirkham *et al.*, 2002).

Despite these findings on high amounts of stored MUC5AC and marked increase in *MUC5AC* gene expression in asthmatic patients, MUC5B has been identified as the principal mucin in mucus plugs from asthmatic patients (Sheehan *et al.*, 1999). Biochemical analysis of a mucus plug from a patient that died in *status asthmaticus* found the mucin content to be over 96 % of the low-charge glycoform of MUC5B (Sheehan *et al.*, 1999; Sheehan *et al.*, 1995). In biopsies from mild asthmatic cases, the main extracellular mucin in the airway lumen forming airway plugs, adjacent to the epithelial lining and in the secretory glandular necks, was found to be MUC5B and not MUC5AC (Groneberg *et al.*, 2002a).

Although much is known about airway mucins in asthma, the role of the gel-forming mucins have not yet fully been determined, leaving speculation open about the possible roles of mucins in equine airway disease such as RAO.

1.6.3 INFLAMMATORY MECHANISMS UNDERLYING HYPER/METAPLASIA OF THE MUCIN PRODUCING CELLS IN HUMAN AIRWAYS

The inflammatory mechanisms in human chronic airway disease that lead to mucus overproduction via mucus-producing-cell hyperplasia and metaplasia have been studied. Much is yet to be learnt about the pathophysiological mechanisms stimulating mucus cell metaplasia and mucus hypersecretion in equine RAO, but it is undoubtedly likely that the mechanisms outlined below which play key roles in human epithelial cell hyper- and metaplasia, particularly in asthma are also involved in equine airway disease.

In asthma there is evidence for both a Th-2 cytokine, and neutrophilic inflammatory-mediators influencing the expression of mucin genes and the number of mucin-producing cells and amount of mucin produced in the respiratory tract. In man, chronic stimulation by Th2-derived cytokines leads to metaplasia of the mucus producing cells in the respiratory tract, and goblet cell hyperplasia has been shown to occur in asthma (Fahy, 2002). Interleukin-4 (IL-4), a Th2-derived cytokine, has been shown to directly induce goblet cell metaplasia and increase *MUC2* gene expression in cultured airway epithelial cells *in vitro* and increased *MUC5AC* gene expression *in vivo* (Dabbagh *et al.*, 1999). Interleukin-9 (IL-9), a Th2-derived cytokine present in asthmatic airway fluid, has been shown to induce *MUC5AC* and *MUC2* gene expression and MUC5AC synthesis in murine models *in vivo* and in cultured airway epithelial cells *in vitro* (Longphre *et al.*, 1999; Louahed *et al.*, 2000). Interleukin-13 (IL-13), another Th2-derived cytokine, has been shown to be essential in the phenotypic expression of murine asthma (Wills-Karp *et al.*, 1998). Neutrophil elastase, a serine protease released from activated neutrophils, has been shown to increase mucus production in neutrophilic inflammatory airway disease via upregulation of *MUC5AC* gene expression (Voynow *et al.*, 1999). In addition to this, neutrophil elastase has also been shown to induce mucus cell metaplasia in murine lung (Voynow *et al.*, 2004).

As we have previously mentioned there is controversy in the immunological basis of RAO, where there is evidence for both Th-2 cytokine and neutrophil mediated inflammatory mediators identified in its pathophysiology; therefore many parallels can be drawn with human asthma. Potentially similar pathways could be influencing the excess mucus seen in the airways of horses with RAO. In the final section of this introduction we will cover what we know so far about mucins, the key structural components of mucus, in

the equine respiratory tract.

1.7 MUCINS IN EQUINE RESPIRATORY TRACT

1.7.1 EQUINE MUCIN GENE EXPRESSION

The first mucin gene to be identified as expressed in equine airways was *eqMuc5ac* (Gerber *et al.*, 2003). Subsequently, *eqMuc5b,* expression as well as Muc5ac protein, has been identified in foal and adult equine airways (Rousseau *et al.*, 2011b; Rousseau *et al.*, 2007). The equine *Muc5b* and *Muc5ac* orthologues of the human MUC genes have been shown to have similarly conserved structure to *MUC5B* and *MUC5AC* (Rousseau *et al.*, 2007). *EqMuc2* expression has been identified in the equine stomach, small intestine and colon, but not, to date, in the respiratory tract (Gerber *et al.*, 2003; Rousseau *et al.*, 2007). *EqMuc5ac* gene expression has also been demonstrated in equine stomach, whilst *EqMuc5b* gene expression has not been investigated and not identified in the equine gastrointestinal tract (Gerber *et al.*, 2003; Rousseau *et al.*, 2011b; Rousseau *et al.*, 2007). The investigations confirming gene expression of *eqMuc5ac* and *eqMuc5b* did not examine epithelium and sub-mucosal glands separately for mucin gene expression (Gerber *et al.*, 2003; Rousseau *et al.*, 2011b; Rousseau *et al.*, 2007). As mentioned previously, in healthy human airways *MUC5AC* is expressed in the epithelial goblet cells whilst *MUC5B* expression is in the sub-mucosal glands, but we currently do not know fully if the equine orthologues are similarly differentially expressed (Buisine *et al.*, 1999).

1.7.2 EQUINE SECRETED POLYMERIC AIRWAY MUCINS

In healthy equine airways, Muc5ac and Muc5b, orthologues to the human airway mucins MUC5B and MUC5AC, have been identified as polymeric mucins in respiratory mucus (Rousseau *et al.*, 2011; Rousseau *et al.*, 2007). Horses differ from man in that Muc5b, rather than MUC5AC appears to be the predominant secreted airway mucin in health (Gerber *et al.*, 2003; Rousseau *et al.*, 2011; Rousseau *et al.*, 2007). The two mucins are polydisperse, high-molecular weight (6-20 MDa), glycosylated proteins joined by disulphide bonds that adopt a stiffened random coil conformation in solution (radius of gyration 140-270 nm) (Rousseau *et al.*, 2007). Both Muc5ac and Muc5b have a heterogenous charge distribution (Rousseau *et al.*, 2011b). Equine Muc5ac exhibits one

broad, electrophoretic band of variable mobility between horses on agarose gel electrophoresis, but analysis by anion-exchange chromatography shows two distinct populations of Muc5ac, with the lower charge density population being the predominate species (Rousseau *et al.*, 2011b). Human MUC5B has two distinct bands identified on agarose gel electrophoresis that can be separated by anion-exchange chromatography into its two glycoforms of low and high charge density (Kirkham *et al.*, 2002). Equine Muc5b also has sub-populations of molecules that can be separated by agarose gel electrophoresis; but contrastingly, anion-exchange chromatography shows one population of equine Muc5b (Rousseau *et al.*, 2011b). The electrophoretic mobility of the equine mucins is therefore, unlike human mucins, not wholly explained by their charge density, indicating that other factors influence their separation on gel electrophoresis, such as molecular weight and extent of glycosylation (Rousseau *et al.*, 2011b).

1.7.3 MUCIN PRODUCING CELLS IN THE EQUINE RESPIRATORY TRACT

Equine respiratory mucins are synthesised in goblet cells of the airway surface epithelium and in mucous cells of sub-mucosal glands, though the extent to which both occur throughout the equine respiratory tract has yet to be fully studied (Gerber *et al.*, 2009b; Hovenberg *et al.*, 1996a; Pirie *et al.*, 1990a; Rousseau *et al.*, 2011b; Wickstrom *et al.*, 1998; Widdicombe and Pecson, 2002). Immunohistochemistry shows that Muc5ac and Muc5b are present in both the respiratory epithelium goblet cells and sub-mucosal glands of the trachea of healthy horses, with a predominance of Muc5b in the goblet cells and Muc5ac in the sub-mucosal glands (Rousseau *et al.*, 2011b). This is in contrast to the situation in human airways where the orthologous mucins, MUC5AC and MUC5B are largely synthesised and stored in separate locations, namely the epithelial goblet calls for MUC5AC and the sub-mucosal glands for MUC5B (Hovenberg *et al.*, 1996a; Wickstrom *et al.*, 1998).

In the following section we review what is currently known regarding the equine airway mucins in airway disease, in order to determine what still needs to be elucidated to fully understand the mucin composition or RAO horse airway mucus.

1.8 MUCINS IN EQUINE AIRWAY DISEASE

1.8.1 MUCIN GENE EXPRESSION IN EQUINE AIRWAY DISEASE

No information on Muc5b expression in RAO is yet available; but gene expression for *Muc5ac* has been shown to be up-regulated in horses with RAO (Gerber *et al.*, 2003). Although *MUC5AC* gene expression is increased in human asthma, MUC5B appears to be the dominant mucin in asthmatic plugs, (refer to *1.6.2*) and as yet we do not know what the situation is for *eqMuc5b* gene expression or mucus mucins present in RAO, hence further investigation of Muc5b and Muc5ac in RAO mucus is warranted. Gene expression for *eqMuc2* has been investigated but not detected in airways of horses suffering from RAO (Gerber *et al.*, 2003; Rousseau *et al.*, 2007).

1.8.2 EQUINE AIRWAYS MUCUS MUCINS IN RESPIRATORY DISEASE

Recent evidence from our laboratory would suggest that in respiratory mucus from racehorses with increased tracheal mucus and increased bacterial counts, there is an increased amount of Muc5b, and in some cases also increases in Muc5ac (Rousseau *et al.*, 2011b). Predominant mucins and relative amounts in mucus from horses with RAO have not been determined, and it is an aim of this PhD project to determine this. As RAO horses have increased tracheal mucus during both symptomatic and asymptomatic states, we intend to investigate mucins in mucus from RAO horses under both conditions.

1.8.3 MUCIN GLYCOSYLATION CHANGES IN EQUINE AIRWAY DISEASE

The glycosylation of mucins has been shown to vary in samples from horses with respiratory disease. Of the five composite sugars that make up the O-linked glycosylated side chains of mucins, significant increases in amounts of fucose and GlcNAc, without significant differences in the amounts of GalNAc or galactose were found in respiratory mucins from challenged (symptomatic) RAO horses compared to those collected from challenged healthy controls (Jefcoat *et al.*, 2001). The levels of sialic acid were also higher in the diseased population but not statistically significant. This indicates that the composition of the glycosylated side chains of mucins from horses with RAO differs to those of horses without RAO. The same study also examined oligosaccharide content of respiratory mucins from the RAO-affected horses after they had been at pasture for 30 days after the initial challenge and found persistent elevations of fucose, GlcNAc and sialic acid compared to challenged control animals, suggesting long-term changes in mucin

glycosylation post-challenge. Unfortunately, longer-term follow-up, or comparison of RAO horses pre-challenge with normal controls pre-challenge was not undertaken. We know that the airway remodeling that occurs during chronic exposure in RAO horses is almost entirely reversible if horses are housed in an open-air non-trigger environment for 12 months or more (Leclere *et al.*, 2012). We do not currently know if the mucin glycosylation changes noted by Jefcoat *et al.* (2001) are a permanent feature of mucins of RAO horses or if they, like the airway remodeling, revert to a healthy-horse mucin glycosylation type if horses are removed from a trigger environment for an extended period of time.

Alterations in mucin glycans could lead to changes in the functional properties of mucus that have important consequences for the protective function of the mucus layer. Glycans provide receptors and also a potential nutritional source for bacteria (Scharfman *et al.*, 1999), the oligosaccharides have important roles in the transport properties of mucus gel, for example fucose has been implicated in the importance of mucus viscoelatic properties (Majima *et al.*, 1999), and they are also targets for pathogen binding (Scharfman *et al.*, 1999). As discussed earlier, equine airway mucins have electrophoretic mobility not fully explained by their charge density, most likely indicating differences in molecular weight possibly due to differences in the extent of glycosylation (Rousseau *et al.*, 2011b). How differences in glycosylation are reflected in biological function still needs to be examined.

1.8.4 EQUINE AIRWAY MUCIN CELLS IN AIRWAY DISEASE

In the horse, inflammation is correlated with an increased amount of secreted mucus within the airways, and a corresponding goblet cell metaplasia and volume of stored mucosubstance (V_s) is observed (Lugo *et al.*, 2006). A large variability in V_s in peripheral equine lung lobes between individual horses is reported, with studies finding greater but not significant differences of V_s and no difference in bronchial goblet cell number or epithelial cell numbers of terminal airways in RAO diseased horses compared to controls (Bartner *et al.*, 2006; Lugo *et al.*, 2006).

1.8.5 INFLAMMATORY MECHANISMS BEHIND MUCUS CELL METAPLASIA IN RAO

There appears to be both neutrophil and Th-2 lymphocyte related mechanisms behind the metaplasia and increased mucin production of inflamed respiratory epithelia as exemplified by experimental animal and human airway cell culture models. In concurrence with these inflammatory processes in other mammals, airway neutrophilia, and evidence of Th2-lymphocyte mediated inflammatory processes are both features found in RAO, as discussed earlier (Lavoie, 2007). Equine *Muc5ac* expression has been shown to be increased by tumour necrosis factor-alpha (TNF- α) in a dose-dependent manner healthy equine epithelial cells in an air-liquid interface culture system; whilst interleukins -2, -4, -5, -8 and -10 in isolation did not have an effect on *eqMuc5ac* mRNA levels (Oslund *et al.*, 2010). An increase in *eqMuc5ac* expression has been found in airway tissues but not BAL fluid of horses suffering from RAO compared to controls (Gerber *et al.*, 2003; Ryhner *et al.*, 2008). Lower IL-13 expression has been seen in the airways fluid from the RAO-affected horses compared to controls (Ryhner *et al.*, 2008).

1.9.1 SUMMARY

Recurrent airway obstruction is a chronic respiratory disease of adult horses that has similarities to human asthma in many ways from clinical signs and pathophysiological mechanisms to chronic pathological lung changes. In both diseases mucus over secretion and reduced clearance are key features contributing to airway obstruction. RAO horses become symptomatic, showing clinical signs on exposure to dusty stable environments, reversible, becoming asymptomatic with return to an open field clean air environment. RAO horses have increased tracheal mucus both whilst symptomatic and whilst asymptomatic, compared to healthy controls. *Table 1.1* summarises the published data reviewed in this introduction on what is known regarding the secreted polymeric airway mucins in human health and asthma and also summarizing what is known and what is yet to be established regarding the secreted polymeric mucins of equine airways. MUC5AC and MUC5B and their orthologues, Muc5ac and Muc5b are the major structural mucins of airways in health, with MUC5AC predominating in human airways, whilst Muc5b

predominates healthy equine airways. In human airways MUC5B and MUC5AC are largely synthesised in separate locations, namely MUC5B in the sub-mucosal glands and MUC5AC in the epithelial goblet cells; with some MUC5B being synthesized in the epithelial goblet cells in asthma. Contrastingly, the mucins appear to be synthesized in both locations in equine airways, and opposite to the human airways, Muc5b is the predominant mucin of goblet cells and Muc5ac is the predominant mucin of sub-mucosal glands (*Table 1.1*). We do know that there is goblet cell hyperplasia in asthma and metaplasia in RAO, but we do not know the full extent or distribution of the mucin producing cells in healthy equine airways, or whether this distribution alters in RAO (*Table 1.1*). MUC5AC is the predominant mucin in secreted mucus in healthy human airways, again the equine airways contrast to this where Muc5b is the predominant mucin of healthy airways mucus (*Table 1.1*). Low and high charge glycoforms of human MUC5B and equine Muc5ac have been identified in mucus from healthy airways (*Table 1.1*).

MUC5AC and MUC5B have been identified as up-regulated in asthma, be it by their gene expression, and or their storage in secretory granules, or their up-regulated secretion into airway fluid and or mucus plugs, and changes in their glycosylated forms (*Table 1.1*). In the highly viscous mucus obstructing the airways of an individual who died in *status asthmaticus*, there were changes in all of these parameters (Sheehan *et al.*, 1999; Sheehan *et al.*, 1995). We know that *eqMuc5ac* mRNA expression is up-regulated in RAO horse airway tissue, but the situation for *eqMuc5b* has not been investigated. It is known that the relative amounts of Muc5b and Muc5ac in equine respiratory mucus can be altered in equine airway disease (IAD), but the situation in RAO is unknown. We do know that the molecular properties of the individual mucins can also be altered in equine airway disease (Jefcoat *et al.*, 2001; Rousseau *et al.*, 2011b). The low-charge glycoform of MUC5B is known to predominate in asthmatic mucus and mucus plugs, but we do not know whether different glycoforms of Muc5b or Muc5ac are involved in RAO mucus (*Table. 1.1*). It is not yet fully known how these alterations present in recurrent airway obstruction, or how they interact to affect and alter the normally protective properties of respiratory secretions.

Table 1.1 **SUMMARY OF PUBLISHED DATA AVAILABLE FOR SECRETORY MUCINS IN HUMAN AND EQUINE AIRWAYS IN HEALTH AND IN ASTHMA/ RAO.** Summary of published findings detailed in *Introduction* on respiratory tissue mRNA gene expression, mucin-producing cell mucin airways distribution and secreted mucin in human and equine airways for **A:** Healthy airways; **B:** Asthma/ RAO airways. (*) Low levels only detected; (?) Unknown; [1] Buisine *et al*., (1999); [2] Vinall *et al*., (2000); [3] Chen *et al*., (2004); [4] Hovenberg *et al*., (1996a); [5] Groneberg *et al*., (2002a); [6] Groneberg *et al*., (2002b); [7] Thornton *et al*., (1990); [8] Davies *e. al*., (1996); [9] Thornton *et al*., (1996b); [10] Thornton *et al*., (1997); [11] Fahy *et al*., (2002); [12] Kirkham *et al*., (2002); [13] Sheehan *et al*., (1995); [14] Sheehan *et al*., (1999); [15] Gerber *et al*., (2003); [16] Rousseau *et al*., (2007); [17] Rousseau *et al*., (2011); [18] Lugo *et al*., (2006).

A

HEALTH	HUMAN	EQUINE
Gene expression (mRNA)	*MUC5AC* expressed in epithelial goblet cells[1] *MUC5B* expressed in sub-mucosal glands[1] *MUC2* expressed in bronchial epithelium*[2] MUC19 expressed in tracheal sub-mucosal glands*[3]	*eqMuc5ac* expressed in tracheal tissue [15,16,17] *eqMuc5b* expressed in tracheal tissue [16,17] *eqMuc2* not expressed in tracheal tissue [15,16,17] ?
Stored glycoprotein	epithelial goblet cells: MUC5AC [4,5,6] sub-mucosal gland ducts: MUC5AC [5,6] sub-mucosal glands: MUC5B [5,6]	epithelial goblet cells: Muc5b > Muc5ac [17] sub-mucosal glands: Muc5ac > Muc5b [17]
Secreted glycoprotien	MUC5AC > MUC5B [7,8,9]	Muc5b > Muc5ac [16,17]
Glycoprotein glycofroms	MUC5B: low and high charge glycoforms [9,10]	Muc5ac: high and low charge glycoform [17]

B

ASTHMA/ RAO	ASTHMA	RAO
Gene expression (mRNA)		*eqMuc5ac* expression increased in tracheal tissue [15] *eqMuc5b* expression ? *eqMuc2* not expressed in tracheal tissue [15]
Stored glycoprotein	epithelial goblet cells: MUC5AC > MUC5B [5] sub-mucosal gland ducts: MUC5AC [5] sub-mucosal glands: MUC5B [5] goblet cell hyperplsia [11]	? ? ? goblet cell metaplasia [18]
Secreted glycoprotein	MUC5AC: increased luminal mucin [5, 12] MUC5B: increased luminal mucin [5,12] MUC5B > MUC5AC [5,13,14]	? ? ?
Glycoprotein glycofroms	MUC5B: low charge > high charge glycoform [14] MUC5B low-charge glycoform from mucus plug branched, cross-linked rather than linear [14]	? ?

1.9.2 HYPOTHESIS

Based on what we know about airway mucins in human asthma and other airway diseases where mucus hypersecretion is part of the pathology, there is likely to be an

increased amount of both mucins Muc5b and Muc5ac correlating with the increased amounts of mucus seen in RAO horse airways. It is also likely that this will relate to an increased amount of both mucins stored in the mucin-producing cells of the airways of RAO horses. MUC5B, which is secreted largely from the sub-mucosal glands predominates in asthma; so the situation in RAO could be that Muc5ac, also produced largely in the sub-mucosal glands, predominates in RAO, or alternatively that Muc5b is the predominant mucin in RAO airways mucus, akin to its human mucin orthologue (MUC5B) in asthmatic airways.

1.9.3 AIMS OF PROJECT

The aims of this project are to attempt to answer the unknown questions regarding the secreted airways mucins in the equine asthma-like disease, RAO. Chiefly we aim to:

- Identify which mucins are present in RAO airway mucus.
- Establish which mucin, if any, predominates.
- Determine where in the equine airways the mucins are produced and the variation between healthy individuals and how this compares to distribution of mucin-producing cells in RAO horse airways.
- Establish if differences occur in mucus mucin-profiles and airway mucin cell distribution of RAO horses when symptomatic and asymptomatic.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

Materials used in multiple experiments are detailed below:

1. Guanidine hydrochloride (GuHCl): Stock solution (approximately 8 M) GuHCl was made from practical grade GuHCl (Sigma-Aldrich, Poole, UK) dissolved by stirring twice overnight in double distilled water with activated charcoal (Sigma-Aldridge, Poole, UK) treatment; first at room temperature and second at 4 ^{o}C. The solution was filtered through 1 x Whatman (GE Healthcare, UK) wet-strengthened and 2 x Whatman qualitative filter papers after each step, to remove the bulk of the charcoal. The solution was then filtered using an Amicon PM-10 ultrafiltration membrane (Millipore, Watford, UK) by connection to water suction vacuum and then finally using an ultrafiltration cell under nitrogen, to remove high molecular weight contaminants prior to use. The molarity of the stock solution was calculated from its refractive index using a refractometer.
2. Nitrocellulose membrane: 0.2 µm pore size Whatman Protran transfer membrane (Whatman, GE healthcare, UK).
3. Wash buffer: Tris buffered saline-Tween (TBST): 10 mM Tris-base, 150 mM sodium chloride (NaCl), 0.1 % (v/v) Tween-20 (Sigma-Aldrich, Poole, UK), pH 8.
4. Densitometer: BioRad GS-800 calibrated densitometer with Quantity One software (BioRad, CA, USA).
5. Urea solution: Stock solution (approximately 6 M) urea was made from practical grade urea (Melford Labs, Ipswich, UK) dissolved by stirring overnight in double distilled water with activated charcoal (Sigma-Aldridge, Poole, UK) and ambilite/mixed bed resin (Sigma-Aldridge, Poole, UK) treatment; at room temperature. The solution was filtered through 1 x Whatman (GE Healthcare, Amersham, UK) wet-strengthened and 2 x Whatman qualitative filter papers after each step, to remove the bulk of the charcoal. The solution was then filtered using an Amicon PM-10 ultrafiltration membrane (Millipore, Watford, UK) by connection to water suction vaccumm prior to use. The molarity of the stock solution was calculated from its refractive index using a refractometer.
6. Dialysis membrane (Medicell International Ltd, London, UK): The dialysis membrane was cut into strips and boiled twice in sodium bicarbonate and EDTA, pH 8 and then stored at 4 ^{o}C in 20 % ethanol (Sigma-Aldrich, Poole, UK).

Membrane was cut to the desired length and washed in double distilled water immediately prior to use.

Table. 2.1 **EQUINE PRIMARY POLYCLONAL ANTIBODIES RAISED IN RABBITS (Rabbit Anti-Equine).** The antibodies were raised using sequences from the cys domains of the respective mucins as follows:

Antibody	**Sequence used to create antibody**	**Antibody: blocking buffer dilution for use**
eqMAN5ac-I (rabbit anti-equine Muc5ac)	GIDSGDFDTLENLR	1:500
eqMAN5b-I (rabbit anti-equine Muc5b)	DEDYPTSEKAGGDIEC	1:100

2.1.1 MUCUS AND MUCIN BIOCHEMICAL ANALYSIS

2.1.1.1 COLLECTION OF MUCUS SAMPLES

1. Clinical samples of airway mucus from RAO patients: Mucus from clinical cases was collected for the purpose of investigation of clinical disease by a veterinary surgeon. We were provided with leftover mucus samples collected by TW or BAL that were not used for disease investigation. Owner informed consent was obtained prior to sample collection and The University of Liverpool's ethics and animal welfare guidelines. Anonymous patient details, and/or a copy of the owner information sheet and example consent form are provided in the *Appendix* (*Appendix 1*: *Tables 1.1,1.2, Figs. 1.1, 1.2*).

2. Tracheal mucus samples from experimental horses: Mucus samples were collected from experimental horses, by our collaborators, at The University of Montreal. The

samples were collected during tracheal endoscopy that was necessary as part of other separate experiments, i.e. no horse underwent endoscopy purely for the purpose of mucus collection for this project. Ethical approval was granted under The University of Montreal's ethics and animal welfare guidelines. Experimental horse details are provided in *Appendix 1 Tables 1.3, 1.4*.

3. 8 M GuHCl.

2.1.1.2 EXTRACTION OF MUCINS

1. 8 M GuHCl.

2.1.1.3 MUCIN REDUCTION AND ALKYLATION

1. Reducing agent: 10 mM Dithiothrietol (DTT; Melford Laboratories, Ipswich, UK).
2. GuHCl reduction buffer: 6 M GuHCl, 0.1 M Tris, 5 M EDTA, pH8.
3. Alkylating agent: 25 mM Iodoacetamide (IAA; Sigma-Aldrich, Poole, UK).

2.1.1.4 MUCIN DETECTION BY SLOT BLOTTING

1. Nitrocellulose membrane.
2. Minifold II 72-well slot blot apparatus (Anderman, Kingston-upon-Thames, UK).

2.1.1.5 PERIODIC ACID-SCHIFF'S (PAS) ASSAY

1. 1 % (v/v) 50 % periodic acid and 3 % (v/v) glacial acetic acid (Thermo Fisher Scientific, UK).
2. 0.1 % (w/v) sodium metabisulphite (BDH, Poole, UK) in 1 mM hydrochloric acid (HCl; Fischer Scientific, Loughborough, UK).
3. Schiff's reagent (Sigma-Aldrich, Poole, UK).
4. Densitometer.

2.1.1.6 IMMUNODETECTION

1. Reducing agent: 25 mM DTT (Melford Laboratories, Ipswich, UK).
2. Washing buffer: Tris-buffered saline-Tween (TBST).
3. Blocking buffer: 4 % (w/v) milk powder (Marvel, UK) in washing buffer.
4. Primary antibodies: Equine primary polyclonal antibodies (raised in rabbits) were diluted in blocking buffer for use at the concentrations given in *Table 2.1*.

5. Secondary antibody: Primary antibodies were detected using goat anti-rabbit IgG alkaline phosphatase (AP)-conjugated secondary antibody (1 mg/ml; Abcam PLC, Cambridge, UK) at a dilution of 1:5000 in washing buffer.
6. The secondary antibody was revealed using the chromogenic substrates 0.4 µM 5-bromo-4-chloroindol-3yl phosphate (BCIP; Thermo Fisher Scientific, UK; 50 mg/mL in dimethylformaldehyde; Sigma-Aldrich, Poole, UK) and 0.4 µM nitro blue tetrazolium (NBT; Melford Labs, Ipswich, UK; 50 mg/mL in 70 % (v/v) dimethylformaldehyde) in alkaline phosphatase buffer.
7. Alkaline phosphatase buffer: 100 mM tris-HCl, 100 mM NaCl, 5 mM magnesium chloride (MgCl), pH 9.5.
8. Densitometer.

2.1.1.7 AGAROSE GEL ELECTROPHORESIS

2.1.1.7a SAMPLE PREPARATION

1. 8 M GuHCl.
2. 6 M urea.
3. 10 x urea reduction buffer (URB): 5 mM Ethylenediaminetetraacetic acid (EDTA; Fisher Scientific, Loughborough, UK), 0.1 M Tris-base (Thermo Fisher Scientific, UK), in 6M urea, pH 8.
4. 10 % (v/v) agarose loading buffer with dye: 10 x URB, 1 % (w/v) sodium dodecyl sulphate (SDS; Thermo Fisher Scientific, UK), 50 % glycerol (v/v) (Thermo Fisher Scientific, UK), and 0.01 % bromophenol blue (Sigma-Aldrich, Poole, UK).
5. Running buffer: 0.1 % (w/v) SDS in 20 mM Tris-base (Thermo Fisher Scientific, UK), 1 mM EDTA (Thermo Fisher Scientific, UK).
6. Reducing agent and buffer: 10 mM DTT (Melford Laboratories, Ipswich, UK) in urea reduction buffer (URB).
7. Alkylating agent and buffer: 25 mM IAA (Sigma-Aldrich, Poole, UK) in URB with 10 % (v/v) agarose loading buffer with dye.

2.1.1.7b ELECTROPHORESIS

1. Agarose gel running buffer: 1 % Tris acetate EDTA (TAE; 40 mM Tris-base (Fisher Scientific, Loughborough, UK), 1 mM EDTA (Thermo Fisher Scientific, UK), pH 8; and 0.1 % (w/v) sodium dodecyl sulphate (SDS).

2. 0.7 % (w/v) electrophoretic grade agarose (Melford Labs, Ipswich, UK) in agarose gel running buffer.
3. Horizontal gel electrophoresis apparatus: Bio-Rad Subcell gel tanks for 25 x 15 cm gels.

2.1.1.7c TRANSFER TO NITROCELLULOSE

1. Nitrocellulose membrane.
2. Vacuum blotting apparatus (Vagugene XL, Pharmacia Biotech) connected to VacuGene pump (GE Healthcare, Amersham, UK) suction at 45-50 mbar.
3. Gel vacuum transfer buffer: 0.6 M NaCl (Thermo Fisher Scientific, UK) and 60 mM sodium citrate (Thermo Fisher Scientific, UK).

2.1.1.7d IMMUNODETECTION

1. Blocking buffer: 4 % (w/v) milk powder (Marvel, UK) in washing buffer.
2. Immunodetection with eqMAN5ac-I and eqMAN5b-I (as for slot-blot protocol in *2.1.1.6*).
3. Densitometer.

2.1.1.8 MUCIN PURIFICATION: ISOPYCNIC DENSITY GRADIENT CENTRIFUGATION IN CAESIUM CHLORIDE (CsCl)/GuHCl

2.1.1.8a SAMPLE PREPARATION

1. 4 M GuHCl.
2. Caesium chloride (CsCl; Melford, Ipswich, UK).

2.1.1.8b SAMPLE CENTRIFUGATION

1. Beckman Quick seal polyallomer centrifuge tubes (100 mL or 40 mL tube volume; Beckman, CA, USA).
2. Beckman fixed angle rotor: Ti70 (100 mL tube volume) or Ti45 (40 mL tube volume) (Beckman, CA, USA).
3. Beckman LM-80 or LK-90 ultracentrifuge (Beckman, CA, USA).

2.1.1.8c PROCESSING DENSITY GRADIENT FRACTIONS

1. Nitrocellulose membrane.

2. PAS assay (refer to *2.1.1.5*).
3. Immunodetection with eqMAN5ac-I and eqMAN5b-I (as for slot blot protocol in *2.1.1.6*).

2.1.1.9 MUCIN IDENTIFICATION BY MASS SPECTROMETRY

1. Reduction agent: DTT (used at 10 mM unless otherwise stated; Melford Laboratories, Ipswich, UK) in GuHCl reduction buffer.
2. Alkylation agent: IAA (used at 25 mM unless otherwise stated; Sigma-Aldrich, Poole, UK) in GuHCl reduction buffer.
3. Dialysis membrane.
4. Sample rehydration in 0.1 M ammonium bicarbonate (Sigma-Aldrich, Poole, UK) and 2 M urea, pH 8.0.
5. Buffer exchange: into 0.1 M ammonium bicarbonate (Sigma-Aldrich, Poole, UK) and 2 M urea, pH 8.0.
6. Vivaspin 100-KDa molecular weight cut-off (MWCO) spin column (Sartorius stedem biotech, Germany).
7. Table top swinging bucket centrifuge (Thermo Fisher Scientific, UK).
8. Digestion: proteomic grade modified trypsin (Promega, CA, USA).
9. Desalt solid phase extraction: C18 ZipTips (Millipore, Massachusetts, USA).
10. Desalt solid phase extraction: Oasis HLB 6 cc Vac Cartridges (Waters, Massachusetts, USA).
11. Elution: 5 % Acetonitrile (Sigma-Aldrich, Poole, UK) in 0.1 % formic acid (Sigma-Aldrich, Poole, UK).
12. 0.1 % formic acid (Sigma-Aldrich, Poole, UK).
13. Speedivac vacuum concentrator with cooling trap (Heto-Holton, Alleroed, Denmark).
14. Tandem mass spectrometer: peptides were separated using a 0.075x250 mm BEH UPLC column on a Waters nanoAcquity system (Waters, Massachusetts, USA), before being automatically analysed on a Thermo LTQ Velos mass spectrometer (Thermo Fisher Scientific, UK).
15. Software: spectra were searched using MASCOT (Matrix Science, UK), and were validated using Scaffold (Proteome Software, OR, USA).

2.1.1.10 RATE ZONAL CENTRIFUGATION

1. Dialysis membrane.
2. 4 M GuHCl.
3. Preformed gradient of 6 to 8 M GuHCl made from 8 M GuHCl stock solution.
4. Beckman polyallomer open top centrifuge tubes (14 mL capacity; Beckman, CA, USA).
5. Beckman SW40 swing out rotor (Beckman, CA, USA).
6. Beckman LM-80 or LK-90 ultracentrifuge (Beckman, CA, USA).
7. Nitrocellulose membrane.
8. PAS staining (refer to *2.1.1.5*).
9. Immunodetection with eqMAN5ac-1 and eqMAN5b-1 were performed (as for slot blot protocol in *2.1.1.6*).

2.1.2 HISTOLOGY

2.1.2.1 TISSUE COLLECTION AND PROCESSING

1. Tissue was collected immediately post mortem from horses fit for human consumption slaughtered at an abattoir.
2. Phosphate buffered saline (PBS; Sigma-Aldrich, Poole, UK).

2.1.2.1a TISSUE FIXATION

1. 10 % (v/v) formalin (Sigma-Aldrich, Poole, UK).
2. 70 % (v/v) ethanol (Sigma-Aldrich, Poole, UK).

2.1.2.1b EMBEDDING

1. Microm Spin Tissue processor (STP) 120 (Microm UK Ltd, UK), following the protocol outlined in *Table 2.2*.
2. Embedding: in paraffin wax blocks: W1 wax pellets (Thermo Shandon, Runcorn, UK) W1 type wax.

Table 2.2 **TISSUE PROCESSING FOR HISTOLOGY.** Tissue samples were processed according to the following pre-set protocol in the Microm STP processor. All chemicals were sourced from Sigma-Aldrich, (Poole, UK) and W1 paraffin wax pellets from Thermo Shandon (Runcorn, UK).

Process	Time
50 % Ethanol	2 minutes
70 % Ethanol	7 hours
90 % Ethanol	45 minutes
95 % Ethanol	3 x 30 minutes
100 % Ethanol	3 x 30 minutes
Xylene	3 x 30 minutes
Paraffin wax	2 hours

2.1.2.2 BLOCK SECTIONING AND SLIDE PREPARATION

1. Micron Cool Cut HM355S microtome.
2. Superfrost polylysine-coated microscope slides: (Thermo Fisher Scientific, UK).

2.1.2.3 SLIDE STAINING

1. Xylene (Sigma-Aldrich, Poole, UK).
2. Industrial methylated spirits (IMS; Sigma-Aldrich, Poole, UK): 100 %, 95 %, 70 %.
3. Double distilled water for periodic acid-Schiff's and alcian blue (PAS-AB) staining.
4. 1 % (w/v) phosphate-buffered saline (PBS; Sigma-Aldrich, Poole, UK) for immunohistochemistry (IHC).

2.1.2.3a PERIODIC ACID-SCHIFFS ALCIAN BLUE (PAS-AB) STAINING

1. Alcian Blue solution: alcian blue 8GX (Sigma-Aldrich, Poole, UK) in 3 % v/v) glacial acetic acid (Thermo Fisher Scientific, UK), filtered and adjusted to pH 2.5.
2. 1 % (v/v) 50 % periodic acid (Thermo Fisher Scientific, UK).
3. Schiff's reagent (Sigma-Aldrich, Poole, UK).

2.1.2.3b IMMUNOHISTOCHEMISTRY

2.1.2.3b.i AFFINITY PURIFICATION OF ANTISERUM

1. Unpurified polyclonal antibodies, eqMAN5ac-I, eqMAN5b-I, (*Table 2.1*).
2. 20 mg Protein-A Sepharose CL-4B beads (GE Healthcare, UK).
3. Binding buffer: 10 mM Tris/ HCl, pH 7.5 (Sigma-Aldrich, Poole, UK).
4. Washing buffer: 0.5 M NaCl (Sigma-Aldrich, Poole, UK) in 10 mM Tris/HCl, pH 7.5 (Sigma-Aldrich, Poole, UK).
5. Acidic Elution buffer: 100 mM glycine, pH 2.5 (Sigma-Aldrich, Poole, UK).
6. Alkaline Elution buffer: 100 mM triethylamine, pH 11.5 (Sigma-Aldrich, Poole, UK).
7. Neutral elution buffer: 10 mM Tris/HCl, pH 8.8 (Sigma-Aldrich, Poole, UK).
8. Neutralisation buffer: 1 M Tris/HCl, pH 8.0 (Sigma-Aldrich, Poole, UK).
9. PBS (Sigma-Aldrich, Poole, UK).
10. 4 M GuHCl.
11. Dry nitrocellulose membrane.
12. TBST.
13. Blocking buffer: 4 % (w/v) milk in TBST.
14. Goat anti-rabbit IgG alkaline phosphatase (AP)-conjugated secondary antibody and BCIP/NBT colour detection method as described previously.

2.1.2.3b.ii POSITIVE AND NEGATIVE CONTROL TISSUE

1. Healthy horse salivary gland and stomach tissue were used as positive and negative control tissues for Muc5ac and Muc5b immunohistochemistry. The tissues were formalin-fixed wax-embedded blocks that had been previously collected and processed for use in an earlier project in our laboratory. Salivary gland was positive control for Muc5b and negative control for Muc5ac; whilst stomach was positive control for Muc5ac and negative control for Muc5b.

2.1.2.3b.iii SLIDE PROCESSING FOR IMMUNOHISTOCHEMISTRY

1. PBS (Sigma-Aldrich, Poole, UK).
2. Microwave

3. 10 mM Sodium Citrate (Sigma-Aldrich, Poole, UK) adjusted to pH 6 with citric acid (Sigma-Aldrich, Poole, UK).
4. Reduction buffer: 10 mM DTT (Melford Laboratories, Ipswich, UK) in 0.1 M Tris/HCl, pH 8 (Sigma-Aldrich, Poole, UK).
5. Alkylation buffer: 25 mM IAA (Sigma-Aldrich, Poole, UK) in 0.1 M Tris/HCl, pH 8 (Sigma-Aldrich, Poole, UK).
6. 3 % H_2O_2 (v/v) (Sigma-Aldrich, Poole, UK) in methanol (Sigma-Aldrich, Poole, UK).
7. Blocking buffer: 10 % donkey serum (Abcam PLC, Cambridge, UK) 1 % bovine serum albumen (BSA: Sigma-Aldrich, Poole, UK) in PBS (Sigma-Aldrich, Poole, UK).
8. Affinity purified primary antibody: used at a dilution of 1:100 (unless otherwise stated) in blocking buffer.
9. Secondary antibody: biotin-conjugated donkey anti-rabbit secondary (donkey anti-rabbit IgG-B: Santa Cruz Biotechnology, Texas, USA), diluted 1:250 in blocking buffer.
10. Immunoperoxidase detection system with avidin and biotinylated horseradish peroxidase (Vectastain Elite ABC System; Vector laboratories, CA, USA).
11. Peroxidase substrate is revealed with 3,3'-Diaminobenzidine (DAB) detection kit (DAB Peroxidase Substrate Kit: Vector laboratories, CA, USA).
12. IMS: 100 %, 95 %, 70 % (Sigma-Aldrich, Poole, UK).

2.1.2.3c COUNTERSTAIN AND MOUNTING

1. Haematoxylin: 1:5 dilution (Sigma-Aldrich, Poole, UK).
2. Fast green stain: 1 % (w/v) fast green (Sigma-Aldrich, Poole, UK) in 1 % (v/v) glacial acetic acid (Sigma-Aldrich, Poole, UK).
3. 1 % (v/v) glacial acetic acid (Sigma-Aldrich, Poole, UK).
4. 100 % IMS (Sigma-Aldrich, Poole, UK).
5. Mounting medium: Xylene based mount (DePex mounting medium: Fisher Scientific UK Ltd, UK).
6. Coverslip (Menzel-Gläser, Thermo Fisher Scientific, UK).

2.1.2.4 IMAGE CAPTURE

1. AxioVision imaging system (microscope, camera and software) version 4.2 (CarlZeiss Ltd, UK).

2.1.2.5 IMAGE ANALYSIS

1. Image analysis was performed using the software package ImageJ (National Institutes of Health, USA).

2.1.3 EQUINE TRACHEAL AIRWAY EPITHELIUM CELL CULTURE

1. Equine airway tracheal epithelial cells were harvested immediately post mortem from healthy horses euthanased for human consumption.

2.1.3.1 CELL HARVESTING METHODS

1. Sigma Dulbecco's phosphate buffered saline (DPBS, without calcium chloride or magnesium chloride (Sigma-Aldrich, Poole, UK).

2.1.3.1a PUBLISHED METHOD OF CELL HARVESTING

1. Cell culture transport media: 50 µg/mL gentamicin (Sigma-Aldrich, Poole, UK), 100 iu/mL penicillin, 0.1 mg/mL streptomycin (PenStrep, Sigma, Poole, UK), and 250 ng/mL amphotericin B (Sigma-Aldrich, Poole, UK) in Dulbecco's modified Eagle's medium (DMEM, with 4500 mg glucose/L, 110 mg sodium pyruvate/L and L-glutamate; Sigma-Aldrich, Poole, UK).
2. Protease for tracheal digestion: 0.2 % (v/v) Protease XIV (Sigma-Aldrich, Poole, UK).
3. Fetal bovine serum (FBS; Gibco Invitrogen, Paisley, UK): 10 % (v/v) in equine cell culture transport media.
4. Equine airway cell culture media: 50:50 mixture of Ham's F12 media (Nutrient mixture F-12 Ham with $NaNCO_3$, with L-glutamine, Sigma-Aldrich, Poole, UK) and DMEM (with 4500 mg glucose/L, 110 mg sodium pyruvate/L and L-glutamate; Sigma-Aldrich, Poole, UK) and the following additives: 5 µg/mL insulin (PromoCell, Heideberg, Germany); 5 µg/mL transferrin (Sigma-Aldrich, Poole, UK); 5 ng/ml epidermal growth factor (EGF; Promokine); 52 µg/mL bovine pituitary extract (BPE; PromoCell, Heideberg, Germany); 40 ng/mL

dexamethasone (Sigma-Aldrich, Poole, UK); 500 µg/mL bovine serum albumin (BSA; Sigma-Aldridge, Poole, UK); 20 ng/mL Choleratoxin from vibriocholerae (Sigma-Aldrich, Poole, UK); 100 iu/mL penicillin and 0.1 mg/mL streptomycin (PenStrep; Sigma-Aldrich, Poole, UK); 50 µg/mL gentamicin (Sigma-Aldrich, Poole, UK); 250 ng/mL amphotericin B (Sigma-Aldrich, Poole, UK).

5. Retinoic acid (RA; Sigma-Aldrich, Poole, UK): Dissolved in molecular grade ethanol (Fisher Scientific, UK) to make a concentrated stock solution, made to 1000 x by adjusting concentration to 50 µM with 1 % (w/v) bovine serum albumin (BSA) in DPBS, stored at -20 ^{o}C.
6. Differentiation media: 15 ng/mL RA added to equine airway cell culture media when cells confluent to encourage differentiation.

2.1.3.1b TRACHEAL EPITHELIUM BRUSHING CELL HARVESTING METHOD

1. Sterile dental brush (Asda, UK).
2. Cell culture transport media.
3. Equine airway culture media.

2.1.3.2 CULTURE OF HARVESTED CELLS

2.1.3.2a PRIMARY CULTURE OF EQUINE AIRWAY CELLS

1. Collagen pre-treatment of culture flasks and transwell plates: bovine collagen solution (Type I: 3 mg/mL PureCol; Advanced Biomatrix, CA, USA) was prepared in advance by dilution 1:80 in PBS (Sigma-Aldrich, Poole, UK). Collagen solution was then added at a sufficient volume to cover the culture surface (flask or trasnwell insert) and left at room temperature overnight. Remaining liquid was then removed and the culture surface washed with PBS (Sigma-Aldrich, Poole, UK) in preparation for use.
2. Collagen pre-coated vented culture flasks (T25 or T75 culture flasks; Corning, NY, USA).
3. Collagen pre-coated air-liquid-interface (ALI) transwell inserts: Corning Costar Transwell Clear (polyester) cell culture inserts, (12 mm diameter 0.4 µm pore size

polyester transwell inserts in 12-well plates; Corning, NY, USA).

2.1.3.2b TRYPSIN TREATMENT TO SEPARATE CELLS

1. Cell culture grade Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldridge, Poole, UK).
2. DPBS (Sigma-Aldrich, Poole, UK).
3. Trypsin neutralizing solution (TNS; Lonza, MD, USA)
4. Equine airway cell culture media.

2.1.3.2c AIR LIQUID INTERFACE CULTURE

1. Cell culture grade Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldridge, Poole, UK).
2. Collagen pre-coated air-liquid-interface (ALI) transwell inserts: Corning Costar Transwell Clear (polyester) cell culture inserts, (12 mm diameter 0.4 µm pore size polyester transwell inserts in 12-well plates; Corning, NY, USA).
3. Equine airway culture differentiation media.

2.1.3.2d APICAL SURFACE WASHING TO REMOVE SECRETED MUCUS FROM ALI CELLS

1. Equine airway cell culture media.

2.1.3.2e FROZEN STORAGE OF CELLS AND DEFROSTING

1. Equine airway transport media containing 10 % (v/v) fetal bovine serum (FBS; Gibco Invitrogen, Paisley, UK) and 10 % (v/v) dimethylsuplhoxide (DMSO; Sigma-Aldrich, Poole, UK).
2. 1 mL cryogenic freezing vials (Cryo vials; Sigma-Aldrich, Poole, UK).
3. Freezing container for freezing vials (Nalgene Mister Frosty, Thermo Scientific, UK).
4. Collagen pre-coated vented culture flasks (T25 or T75 culture flasks; Corning, NY, USA).
5. Equine airway culture media.

2.2 METHODS

2.2.1 MUCUS AND MUCIN BIOCHEMICAL ANALYSIS

2.2.1.1 COLLECTION OF MUCUS SAMPLES

In clinical cases and experimental horses, equine airway secretions were collected (on our behalf) via tracheal wash (TW) or bronchoalveolar lavage (BAL) during endoscopic examination of airways, by infusion of sterile 0.9 % saline into the airways and subsequent collection via suction through sterile endoscopic tubing. Once collected, TW and BAL samples were mixed with an equal volume of 8 M GuHCl (to prevent proteolytic degradation) and refrigerated prior to analysis.

2.2.1.2 EXTRACTION OF MUCINS

Mucus, mixed with 8 M GuHCl as described above, was solubilised by gentle mixing at 4 ^{o}C for at least 24 hours.

2.2.1.3 MUCIN REDUCTION & ALKYLATION

Mucus samples were reduced with 10 mM DTT in 6 M GuHCl reduction buffer at 37 ^{o}C for 1.5 hours, followed by alkylation of thiol groups to prevent disulphide bond re-formation with 25 mM IAA in the same buffer in the dark at room temperature for 20 minutes.

2.2.1.4 MUCIN DETECTION BY SLOT BLOTTING

Glycoproteins were blotted (25 to 100 µL depending on sample, refer to experimental *Chapters 3* and *5*) onto a pre-wetted nitrocellulose membrane in slot-form via vacuum suction using slot blot apparatus. Glycoproteins were revealed using either periodic acid-Schiff's staining (refer to *2.2.1.5*) or by immunodetection (refer to *2.2.1.6*) and then density measured by use of a densitometer and imaging software.

2.2.1.5 PERIODIC ACID-SCHIFF'S (PAS) ASSAY

Glycoprotein blotted onto nitrocellulose membrane was revealed using the periodic

acid-Schiff's assay. The membrane was incubated for 30 minutes at room temperature in 1 % (v/v) periodic acid and 3 % (v/v) acetic acid, washed with water and then incubated for 30 minutes in 0.1 % (w/v) sodium metabisulphite before incubation with Schiff's reagent until colour change was observed, then rinsed in 0.1 % (w/v) sodium metabisulphite, and washed in double distilled water, before drying the membrane (Thornton *et al.*, 1996a).

2.2.1.6 IMMUNODETECTION

Immunodetection of mucin after slot blotting (*2.1.1.4*) or vacuum blotting (*2.2.1.7c*) to nitrocellulose membrane firstly involved reduction of non-reduced blotted samples with 10 mM DTT in washing buffer, and then all membranes were blocked in blocking buffer for 30 minutes prior to primary antibody application. Immunodetection was then carried out by incubation with primary antibody for either equine Muc5ac or equine Muc5b followed by alkaline-phosphatase based detection (refer to *2.2.1.6a*; *Fig. 2.1*).

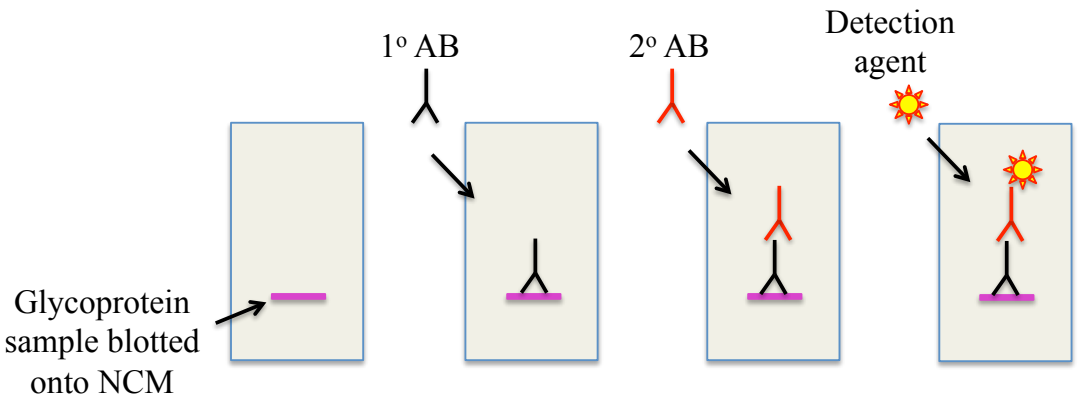
Both equine primary polyclonal antibodies were raised in rabbits using sequences from the cys domains of the respective mucins and used diluted in blocking buffer (*Table 2.1*).

Following overnight incubation with primary antibody, membranes were washed with washing buffer before incubating with secondary alkaline phosphatase antibody.

2.2.1.6a ALKALINE PHOSPHATASE ANTIBODY DETECTION

Goat anti-rabbit IgG phosphatase conjugated antibody (1 mg/ml) was incubated at a 1/5000 dilution with the membrane at room temperature for 30 minutes, after which the membrane was washed with TBST prior to visualisation. Secondary antibody was detected by incubation of the membrane with 0.4 μM BCIP and 0.4 μM NBT in alkaline phosphatase buffer until a colour change occurred, at which point the reaction was stopped in double distilled water and then the membrane was dried (*Fig. 2.1*).

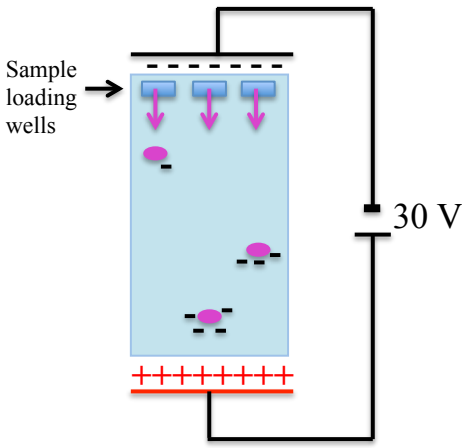
Fig. 2.1 **SCHEMATIC DIAGRAM OF IMMUNODETECTION STAINING**. Glycoproteins adherent to nitrocellulose membrane (via Western blot following agarose gel electrophoresis or via slot-blotting) are probed for primary antibody (1° Ab) reactivity and incubated with conjugated secondary antibody (2° Ab) and revealed using visible detection agent substrates (diagram not to scale).



2.2.1.7 AGAROSE GEL ELECTROPHORESIS (A.G.E.)

Agarose gel electrophoresis was employed to separate the large mucin glycoproteins based on a combination of their molecular weight and negative charge (Thornton *et al.*, 1996a) (*Fig.2.2*).

Fig. 2.2 **SCHEMATIC DIAGRAM OF AGAROSE GEL ELECTROPHORESIS.** Samples are loaded into sample wells at negative side of circuit in horizontally positioned 0.7 % agarose gel in submersion apparatus. A potential difference of 30 V is passed through the gel for 16.5 hours. Glycoproteins (represented by pink oval in figure) move differing amounts through the gel towards the positive electrode depending on their negative charge (diagram not to scale).



2.2.1.7a A.G.E.: SAMPLE PREPARATION

Samples in 4 M GuHCl were buffer exchanged by dialysis into 6 M urea prior to agarose gel electrophoresis. If samples had previously been reduced and alkylated, 10 % (v/v) agarose loading buffer with dye was added to samples in preparation for loading into the gel wells. Non-reduced samples were reduced and then alkylated to prevent reformation of disulphide bonds. Samples were reduced with 10 mM DTT in URB, at 37 ^{o}C for 1.5 hours; then alkylated, with alkylating agent and buffer (25 mM IAA in URB with 10 % (v/v) agarose loading buffer with dye) in the dark for 20 minutes at room temperature.

2.2.1.7b A.G.E.: ELECTROPHORESIS

0.7 % (w/v) agarose gels were loaded and electrophoresis performed in the same running buffer in an horizontal gel apparatus at 30 V for 16.5 hours at room temperature (Sheehan *et al.*, 2000; Thornton *et al.*, 1996a) (*Fig. 2.2*).

2.2.1.7c A.G.E.: TRANSFER TO NITROCELLULOSE

Following electrophoresis, glycoproteins were transferred onto nitrocellulose membrane via vacuum suction at 45-50 mbar (Vagugene XL, Pharmacia Biotech) for 1.5 hours in 0.6 M NaCl and 60 mM sodium citrate.

2.2.1.7d A.G.E.: IMMUNODETECTION

After vacuum transfer of electrophoresed gel samples, nitrocellulose membrane was blocked in 4 % (w/v) milk in TBST for 30 minutes prior to addition of primary antibody. Refer to section *2.2.1.6* for details of antibody mediated mucin detection.

2.2.1.8 MUCIN PURIFICATION: ISOPYCNIC DENISTY GRADIENT CENTRIFUGATION IN CAESIUM CHLORIDE (CsCl)/GuHCl

Two consecutive density gradient centrifugation steps were used to purify mucins from nucleic acids and non-mucin proteins based on their differential buoyant densities in GuHCl (Carlstedt *et al.*, 1983b).

2.2.1.8a CsCl DENSITY GRADIENT: SAMPLE PREPARATION

To separate proteins from mucins the mucus extracts were centrifuged in a 4 M

GuHCl/ CsCl density gradient, the starting density (Þ) was set at 1.4 g/ mL. Subsequently, to remove nucleic acids (and remaining proteins) a second CsCl density gradient was performed at a starting density of 1.5 g/ mL in 0.2 M GuHCl.

The GuHCl concentration and the amount of CsCl to be added to the solubilised mucus samples were calculated as follows:

The refractive index (n) of a sample is measured on a refractometer then the GuHCl molarity of the sample is calculated based on the following equation:

$$M = 60.4396n - 80.5495$$

The amount (χ) of CsCl to be added to each sample was calculated as follows:

$$\chi = \alpha\ [\ (1.347Þ) - (0.0318M) - 1.347]$$

Where:

α = final volume of solution to be centrifuged in mL

Þ = final density of solution to be centrifuged in g/ mL M = molarity of sample

The calculated amount of CsCl (χ) was weighed out and then the sample was added to up to the final weight, calculated as follows:

$$\text{Final weight (g)} = Þ \times \alpha$$

2.2.1.8b CsCl DENSITY GRADIENT: SAMPLE CENTRIFUGATION

Once the CsCl was dissolved in the weighed sample, the sample was added to a Beckman centrifuge tube and then heat-sealed. Balanced, paired centrifuge tubes were spun in a Beckman LK-90 centrifuge in a Ti70 (100 mL tube volume) or Ti45 (40 mL tube volume) rotor, at 40,000 rpm (285,000 x g) for 68 to 72 hours at 15 ^{o}C.

2.2.1.8c CsCl DENSITY GRADIENT: FRACTION PROCESSING

Twenty fractions were unloaded from the bottom of the centrifuge tube; 2 mL fractions from 40 mL tubes or 5 mL fractions from 100 mL tubes. Density (g/ mL) and optical density (at 280 nm) of each fraction were then measured. An aliquot of each fraction was slot-blotted onto nitrocellulose membrane and then PAS assay (refer to

2.2.1.5) and immunodetection with eqMAN5ac-I and eqMAN5b-I (refer to *2.2.1.6*) were performed.

2.2.1.9 MUCIN IDENTIFICATION BY MASS SPECTROMETRY

2.2.1.9a MASS SPECTROMETRY: SAMPLE PREPARATION

Fractions identified as mucin-containing from analysis of the CsCl/ GuHCl density gradient centrifugation were pooled, and an aliquot reduced and alkylated and prepared for mass spectrometry. Samples had the CsCl and GuHCl removed by either a process of water dialysis and freeze-drying and subsequent rehydration in 0.1 M ammonium bicarbonate and 2 M urea, pH 8.0; or by buffer exchange into 0.1 M ammonium bicarbonate and 2 M urea, pH 8.0 by spinning the samples through a 100-KDa MWCO spin column at 5000 rpm. Samples were digested overnight at 37 °C with an excess (1 µg) of modified trypsin. The trypsin-digested sample was then desalted (to remove any urea or ammonium bicarbonate salts that may interfere with mass spectrometry) using solid phase extraction (with C18 ZipTips or Oasis HLB 6 cc Vac Cartridges) and the peptides were eluted with 50 % acetonitrile in 0.1 % formic acid. The acetonitrile was evaporated in a vacuum to concentrate tryptic peptides in 0.1 % formic acid.

2.2.1.9b MASS SPECTROMETRY

The eluted digested peptides were separated by reverse-phase chromatography and analysed in-line by positive ion electrospray ionisation tandem mass spectrometry (MS/MS). The peptides were separated using a 0.075 x 250 mm BEH UPLC column on a Waters nanoAcquity system before being automatically analysed on a Thermo LTQ Velos mass spectrometer. Raw data was processed to produce MASCOT general files (*.mgf). The mgf files produced were searched against a custom database containing SwissProt database (EBI) and predicted sequences of the five equine secreted gel-forming mucins (Muc2, Muc5b, Muc5ac, Muc6 and Muc19) using MASCOT software (Matrix Science). The equine mucin sequences within the database were predicted from the most recent equine genome assembly (EquCab2). Parameters for the MASCOT search were: trypsin cleavage, carbamidomethyl-cysteine and methionine oxidation, allowing for 1 missed cleavage, 2+ and 3+ charges and tolerance levels of +/- 1.2 Da and +/- 0.6 Da for peptide and fragment masses.

2.2.1.10 MUCIN SIZE SEPARATION BY RATE ZONAL CENTRIFUGATION

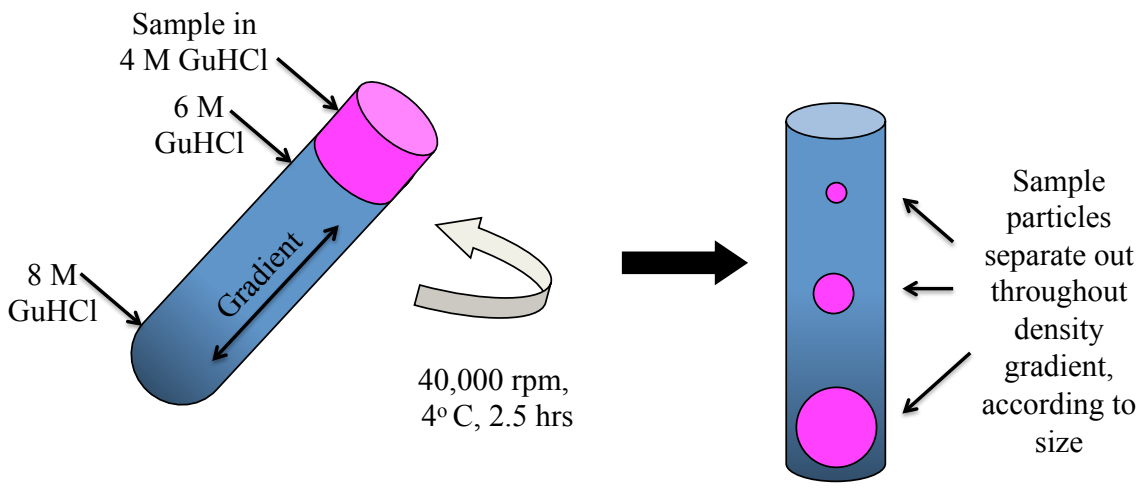
Mucins can be separated according to size (assuming an identical mucin shape) by centrifugation through a pre-formed density gradient, known as rate zonal centrifugation (*Fig. 2.3*). Solubilised mucus samples were dialysed into 4 M GuHCl prior to rate zonal centrifugation. Samples were loaded on top of a preformed gradient of 6 to 8 M GuHCl and centrifuged in a Beckman SW40 swing out rotor in a Beckman L-80 centrifuge at 40,000 rpm at 15 ^{o}C for 2.5 hours. If samples were reduced and alkylated prior to rate zonal centrifugation, centrifugation time was extended to 7 hours.

Following centrifugation, 24 fractions were unloaded from the top of the tube (24 x 0.5 ml fractions from a 12 ml tube). The refractive index (n) of each fraction was measured on a refractometer then the GuHCl molarity of the sample calculated based on the following equation:

$$M = 60.4396n - 80.5495$$

An aliquot of each fraction was slot blotted onto nitrocellulose membrane. PAS staining and immunodetection with eqMAN5ac-1 and eqMAN5b-1 were performed (refer to sections *2.2.1.5, 2.2.1.6*).

Fig. 2.3 **SCHEMATIC REPRESENTATION OF RATE ZONAL CENTRIFUGATION.** Samples (pink) in 4 M GuHCl are layered on top of a pre-formed 6 to 8 M GuHCl gradient (blue) and are centrifuged (40,000 rpm at 15 ^{o}C in a Beckman SW40 swing out rotor for 2.5 hours), separating sample particles on the basis of size throughout the gradient. (Diagram not to scale).



2.2.2 HISTOLOGY

Tissue samples for the histological study of the number and distribution of mucin-producing cells within the respiratory tract epithelium and sub-mucosal glands of healthy horses were collected and processed as follows.

2.2.2.1 TISSUE COLLECTION AND PROCESSING

Tissue was collected immediately post mortem from horses fit for human consumption slaughtered at an abattoir. The trachea and lungs were removed intact from the carcass and then sections taken as detailed below and transported (for approximately 2 hours) in PBS on ice to the processing centre where they were sectioned and fixed prior to histological processing.

2.2.2.1a RESPIRATORY TRACT SECTIONS

The number of tracheal rings was counted and found to be 50 in every horse studied. Segments containing 3-tracheal rings in longitudinal length were taken from the cranial, middle and distal trachea by sharp dissection. Specifically rings 3, 4, 5 for the cranial section, rings 24, 25, 26 for middle section and rings 45, 46, 47 for the caudal section (*Figs. 2.4, 2.5*). The bronchial tree for one lung lobe was dissected out and sections of primary, secondary and tertiary bronchus collected (*Figs. 2.4, 2.5*).

2.2.2.1.b TISSUE FIXATION

Equal amounts of each tissue were fixed in either 10 % formalin or 70 % ethanol. In brief, the 3-ring wide tracheal sections were dived through the central tracheal ring, making two 1.5-ring wide sections. Then each of the 1.5-ring sections was divided longitudinally into quarter sections: dorsal, ventral, left and right (*Fig. 2.5*) and then fixed. The primary, secondary and tertiary bronchi were divided in two, width-ways to preserve their tubular longitudinal structure.

2.2.2.1.c EMBEDDING

Once tissues had been fixed in formalin or ethanol for more than 3 days, 5 to 8 mm wide cross-sections of representative pieces of tissue were processed overnight in steps of ethanol, xylene and finally wax immersion, in an automated processor and then embedded in paraffin wax blocks.

Fig. 2.4 **PHOTOGRAPHS DEMONSTRATING RESPIRATORY TRACT TISSUE SECTIONS TAKEN FOR HISTOLOGICAL STUDY OF HEALTHY EQUINE AIRWAYS.** **A**. Photograph of fresh post-mortem healthy equine respiratory tract. Superimposed black brackets demonstrate position of cranial, middle and caudal sections of 3-tracheal ring sections taken for histological study (used in *Chapter 4*). **B.** Photograph of dissected fresh post-mortem healthy equine bronchial tree with circles demonstrating position of primary, secondary and tertiary bronchus sections taken for histological study (used in *Chapter 4*).

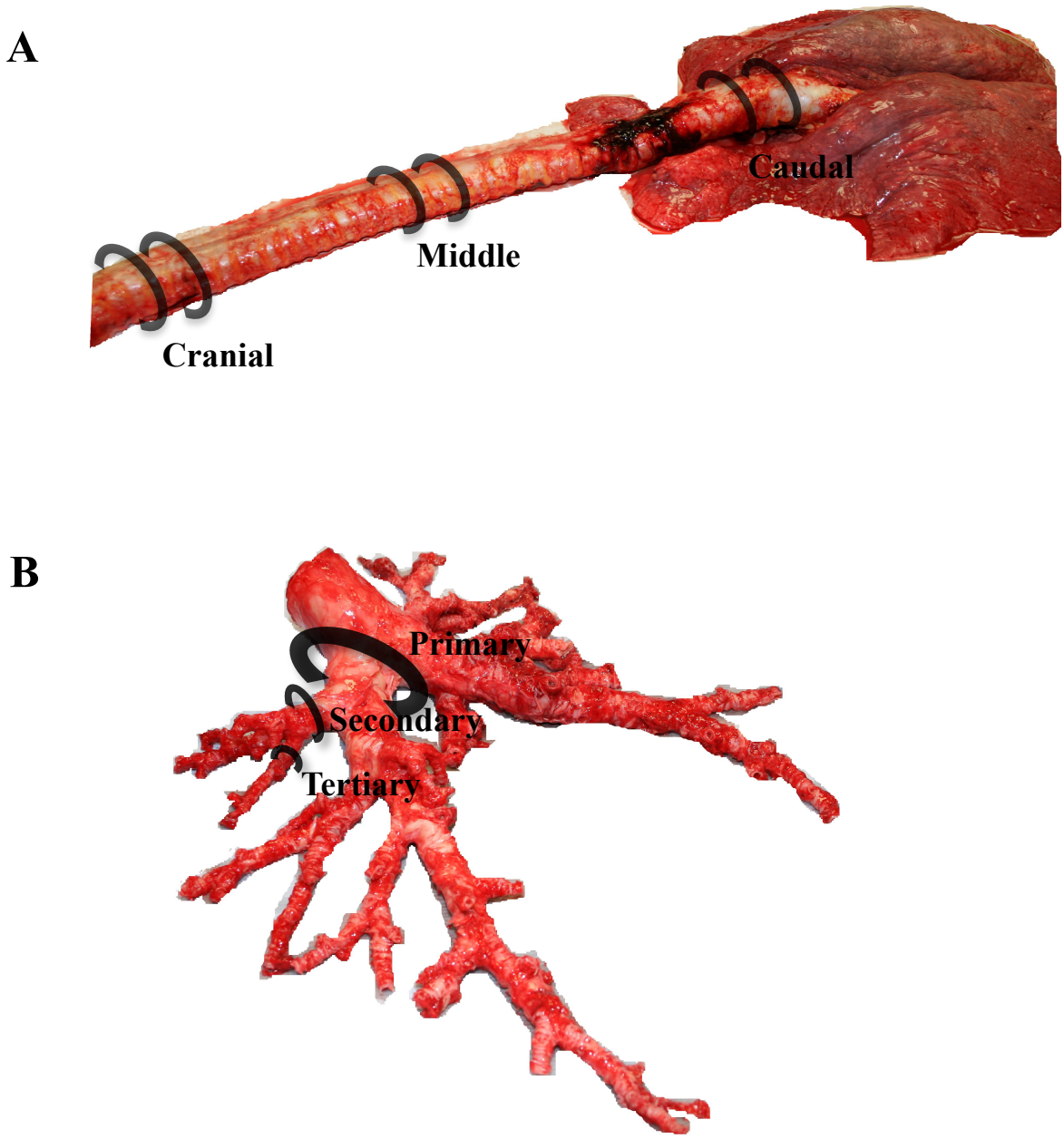
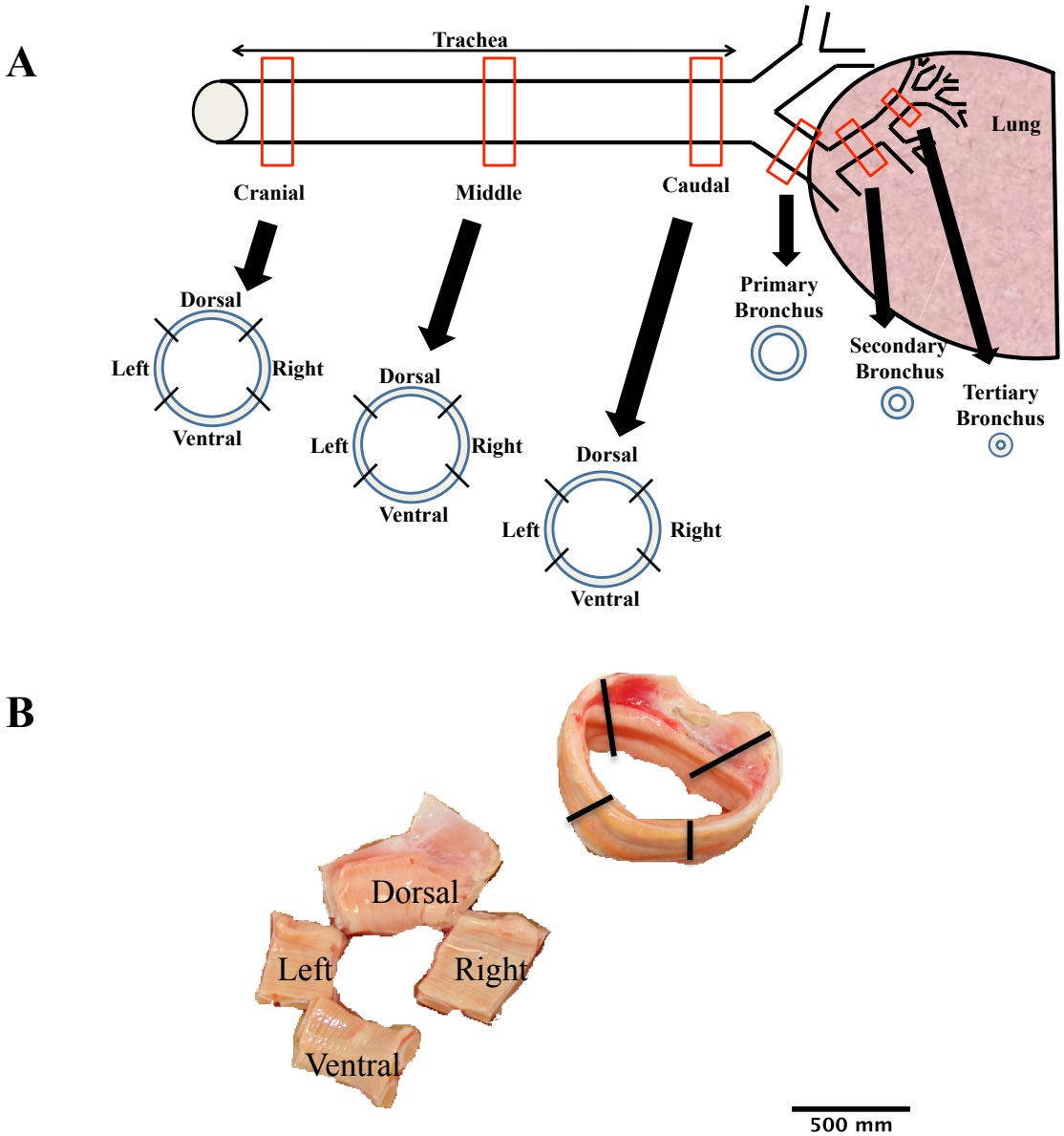


Fig. 2.5 **ILLUSTRATION OF RESPIRATORY TRACT TISSUE SECTIONS FOR HISTOLOGICAL STUDY OF HEALTHY EQUINE AIRWAYS.** **A:** Schematic diagram of anatomical location of sections taken from each healthy horse respiratory tract for histological study (used in *Chapter 4*). For each area sectioned, identified by red boxes, duplicate pieces of tissue were fixed in 10 % formalin and 70 % ethanol, therefore doubling the number of sections. Tracheal ring sections harvested from the cranial, middle and caudal trachea were divided into dorsal, ventral, left and right sections, as demonstrated in **A:** (diagram not to scale) and **B:** Photograph of cross section of equine trachea with black lines demonstrating the lines of cut used to produce 4 quarter sections of the trachea for fixation, labelled in the photograph of the cut tracheal section.



2.2.2.1.d BLOCK SECTIONING AND SLIDE PREPARATION

Three x 5 micron serial sections (for staining with general mucin stain PAS-AB and immunohistochemistry with eqMuc5b and eqMuc5ac; refer to *2.2.2.2*) of tissue were cut from the wax blocks using a microtome, floated in a warm water bath and laid on polylysine-coated microscope slides and dried at 37 ^{o}C overnight.

2.2.2.2 SLIDE STAINING

Prior to staining, slides with wax-tissue sections dried on were de-waxed in xylene at room temperature for 10 minutes followed by a further 5 minutes in fresh xylene; then washed in 100 % IMS (industrial methylated spirits) for 10 minutes, 95 % IMS for 5 minutes, then 70 % IMS for 5 minutes and then rehydrated with water (for Alcian Blue and PAS stain) or 1 % PBS (for immunohisotchemistry) prior to staining.

2.2.2.2a PERIODIC ACID-SCHIFFS AND ALCIAN BLUE (PAS-AB) STAINING

Slides were immersed in Alcian Blue (AB) (pH 2.5) stain for 5 minutes before washing in water. The slides were then immersed in 1 % periodic acid in water at room temperature for 5 minutes and then washed in water before addition of Schiff's reagent for 5 minutes. Slides were then washed in running water for 10 minutes. Slides were then counterstained with haematoxylin (refer to *2.2.2.2c*) and then dehydrated in 100 % IMS before mounting (refer to *2.2.2.2c*).

2.2.2.2b IMMUNOHISTOCHEMISTRY

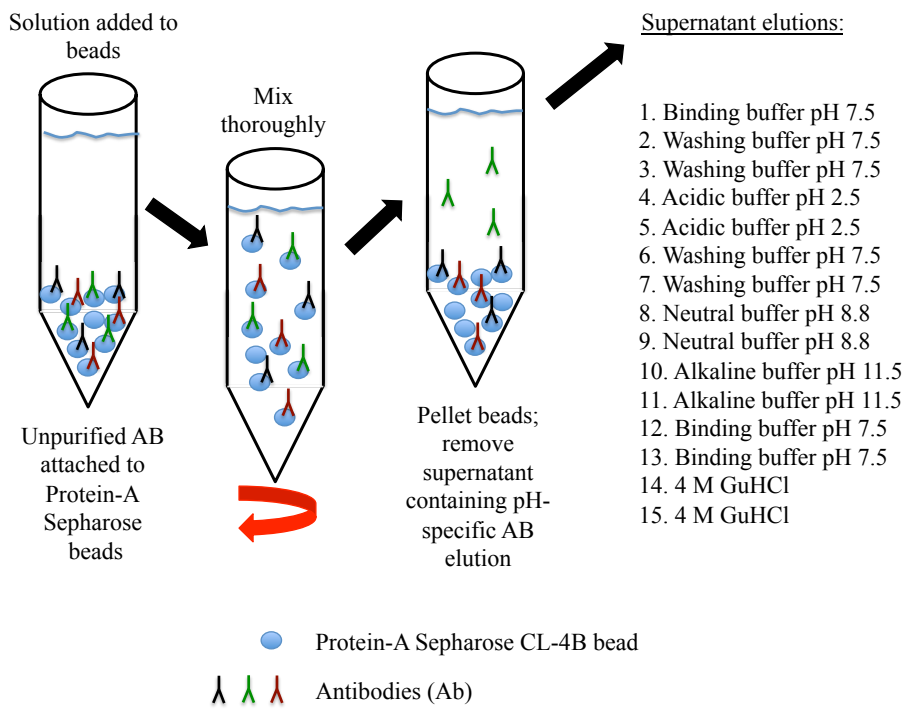
The equine respiratory mucin (Muc5b and Muc5ac) antibodies were affinity purified and tested for tissue specificity prior to their use in immunohistochemistry (*Fig. 2.6*).

2.2.2.2b.i AFFINITY PURIFICATION OF ANTISERUM

Unpurified polyclonal antibodies (eqMAN5ac-I, eqMAN5b-I) were purified as has been described previously via binding of unpurified antibody to Protein-A Sepharose beads and subsequent elution of antibody at varying pH solutions (Rousseau *et al.*, 2011b). In brief, 20 mg Protein-A Sepharose CL-4B beads were washed in 1 mL PBS and rotated for one hour (at 4 ^{o}C) then pelleted and the supernatant discarded. The washing procedure was repeated twice more and the pellet was re-suspended with the unpurified antibody diluted

1:5 in binding buffer and mixed at 4 °C overnight. The beads were then pelleted by centrifugation and the supernatant removed and kept for further processing (fraction 1). The pellet was washed with 0.5 mL washing buffer twice and the supernatant removed and kept after each wash (fractions 2 & 3). The pellet was then twice re-suspended with 0.5 mL acidic elution buffer and then re-pelleted and the supernatant removed and added to 50 µL neutralisation buffer (fractions 4 & 5). The pellet was washed twice with 0.5 mL of washing buffer, with 50 µL of neutralisation buffer being added to the supernatants (fractions 6 & 7). The pellet was then re-suspended twice with the neutral buffer and 50 µL of neutralisation buffer added to the supernatants (fractions 8 & 9). The pellet was then twice re-suspended with 0.5 mL alkaline elution buffer and then re-pelleted and the supernatant removed and added to 50 µL neutralisation buffer, (fractions 10 & 11). The pellet was then twice re-suspended with 0.5 mL binding buffer and then re-pelleted and the supernatant removed and added to 50 µL neutralisation buffer (fractions 12 & 13). The pellet was then twice washed with 0.5 mL 4 M GuHCl and then re-pelleted and the supernatant removed and added to 50 µL neutralisation buffer (fractions 14 & 15) (*Fig. 2.6*).

Fig. 2.6 **SCHEMATIC DIAGRAM REPRESENTING ANTIBODY (AB) AFFINITY PURIFICATION.** Unpurified equine polyclonal antibody was adhered to 20 mg ProteinA Sepharose CL-4B beads and then eluted at varying pH solutions, outlined in figure, to create 15 separate supernatant fractions.



The fractions (1-15) were tested for presence of antibody by dot blotting 5 µL of each elution onto dry nitrocellulose membrane and allowing to dry fully before wetting in TBST and blocking in 4 % milk for one hour before secondary antibody incubation and detection using the BCIP/NBT method as described in *2.2.1.6a*. Fractions that had a positive BCIP/NBT signal detection were used on positive and negative control tissue sections for determination of mucin-tissue specific antibody tissue staining.

2.2.2.2b.ii IMMUNOHISTOCHEMISTRY (IHC) DAB DETECTION

In brief, IHC slide preparation involved washing dewaxed rehydrated slides followed by an antigen retrieval step, reduction and alkylation, quenching of endogenous peroxidase activity, blocking of protein within tissue prior to incubation with the affinity-purified primary antibody and subsequent secondary antibody application and detection using 3,3'-Diaminobenzidine (DAB) and counterstaining of background tissues.

2.2.2.2b.iii SLIDE PROCESSING FOR IMMUNOHISTOCHEMISTRY

Dewaxed, rehydrated (in PBS) slides were washed in PBS, 3 times for 5 minutes, before the antigen retrieval step of microwaving slides immersed in 10 mM Sodium Citrate at pH 6 (adjusted with citric acid) for 10 minutes. The antigen retrieval step was omitted in some experiments, as detailed in *Chapter 4*. Slides were allowed to cool before reduction with 10 mM DTT in 0.1 M Tris/HCl, pH 8 at room temperature for 30 minutes, followed by alkylation with 25 mM iodoacetamide in 0.1 M Tris/HCl, pH 8 in the dark, at room temperature for 30 minutes. Slides were then washed with PBS for 3 x 5 minutes. Endogenous peroxidase activity was quenched with 3 % H_2O_2 (v/v) in methanol for 30 minutes before blocking in blocking buffer (10 % donkey serum 1 % BSA in PBS) for one hour. Affinity purified primary antibody was applied to slides at a dilution of 1:100 (unless otherwise stated) in blocking buffer, and incubated at 4 ^{o}C overnight.

Slides were washed with PBS for 3 x 5 minutes before incubation with secondary antibody (biotin-conjugated donkey anti-rabbit secondary) diluted 1:250 in blocking buffer for 30 minutes. Slides were then washed with PBS for 3 x 5 minutes before incubation with 200 µL/ tissue section of an avidin-peroxidase kit for 30 minutes. Slides were then washed with PBS for 3 x 5 minutes before incubation with a DAB detection kit (200 µL/ tissue section) for 25 minutes to reveal mucin staining. Slides were washed with PBS and then counterstained (refer to *2.2.2.2c*). Following counterstaining, IHC slides were

dehydrated in 100 % IMS (industrial methylated spirits) for 10 minutes, 95 % IMS for 5 minutes, then 70 % IMS for 5 minutes and mounting (refer to *2.2.2.2c*).

2.2.2.2c COUNTERSTAIN AND MOUNTING

Slides were either counterstained with fast green or haematoxylin. Counterstaining with haematoxylin (1:5 dilution) was performed for 30 seconds before immersion in warm tap water for 2 minutes. Alternatively (refer to *Chapter 4* for details) slides were counterstained with fast green (1 % (w/v) in glacial acetic acid, unless otherwise stated) for 1 minute followed by washing in water and then 1 % (v/v) glacial acetic acid before dehydration and mounting.

Slides were mounted using xylene based mounting medium and sealed with a coverslip.

2.2.2.3 IMAGE CAPTURE

Images of sections of epithelium were captured using a x 20 objective lens on a AxioVision (version 4.2; CarlZeiss Ltd, UK) imaging system combined microscope, camera and software package.

2.2.2.4 IMAGE ANALYSIS

Images were analysed using a software package (ImageJ; National Institutes of Health, USA). The scale of the image was calibrated to pixel count. For each x 20 image, epithelium basal lamina length was measured by drawing a freehand line along the length of the epithelium in the ImageJ software package and then measuring it. Epithelium total area was measured by outlining the epithelial area using the freehand drawing tool, selecting that area and then measuring. Epithelial goblet cells were selected using colour threshold technology within the ImageJ software package, and then the number of goblet cells staining positive for mucin within the total epithelium on the image were counted and the total mucin-positive goblet cell area measured. The number of goblet cells per unit length of epithelium (1000 μm for this study) and the average goblet cell size were then calculated using the following calculations:

$$\text{Goblet cell number per unit length epithelium} = \frac{\text{Goblet cell count}}{\text{Epithelium length } (\mu m)} \times 1000\ \mu m$$

$$\text{Average goblet cell size per image} = \frac{\text{Goblet cell total area}}{\text{Goblet cell number}}$$

In images where glandular tissue was present, gland total area was outlined by drawing a freehand elipse around the gland tissue and then the total mucin-stain positive area selected and measured using colour threshold technology. The percentage of gland occupied by mucin-positive stain was calculated:

$$\text{\% mucin positive area of gland} = \frac{\text{Mucin stain positive gland area}}{\text{Total gland area}} \times 100$$

2.2.2.5 STATISTICAL ANALYSIS

Statistical analysis and graphs of the data were done using the computer software packages Minitab and GraphPad Prism. Significance was classed as $P < 0.05$.

2.2.3 EQUINE TRACHEAL AIRWAY EPITHELIUM CELL CULTURE

Equine airway tracheal epithelial cells were harvested immediately post mortem from healthy horses euthanased for human consumption. Epithelial cells were prepared for cell culture following the previously published method (Oslund *et al.*, 2010; Schwab *et al.*, 2010) involving enzymatic digestion of a section of tracheal rings prior to harvesting of epithelial cells; or by collection of epithelial cells via brushing the epithelium using a sterile dental brush immediately post mortem.

2.2.3.1 CELL HARVESTING METHODS

Immediately following euthanasia of healthy donors, the trachea was dissected out and gently rinsed with cold Dulbecco's phosphate buffered saline (DPBS) to remove blood and debris.

2.2.3.1a PUBLISHED METHOD OF CELL HARVESTING

Whole tracheal sections of 10 tracheal rings in length were immersed in cell culture transport media and transported on ice to the laboratory for further processing. Once transported, the tracheal sections were enzymatically digested for > 48 hours in cell culture

transport media containing 0.2 % (v/v) proteases. The tracheal epithelium was then scraped using a sterile scalpel blade to collect epithelial cells. Cells were re-suspended in culture transport media containing 10 % (v/v) FBS and then pelleted by centrifugation (3000 rpm for 3 minutes) before re-suspending in equine airway culture media.

2.2.3.1b TRACHEAL EPITHELIUM BRUSHING CELL HARVESTING METHOD

Rinsed post mortem trachea sections were aseptically opened longitudinally using a scalpel blade. A sterile dental brush was used to collect epithelial cells by repeatedly applying the brush to the tracheal epithelial surface either circumferentially and longitudinally (refer to *Chapter 5*). Brushes were then immersed in 10 mL cell culture transport media on ice and transported to the laboratory for processing. Cells collected via brushing were removed from the brushes in the transport media by gentle repeated lavage of the brush using a sterile Pasteur pipette. Once the brush head was visibly clear of cellular debris, the brush was removed and the solution centrifuged (3000 rpm for 3 minutes) to pellet the cells. The cells were then re-suspended in equine airway culture media.

2.2.3.2 CULTURE OF HARVESTED CELLS

All cultures were grown in a humidified 5 % CO_2 atmosphere incubator at 37 ^{o}C.

2.2.3.2a PRIMARY CULTURE OF EQUINE AIRWAY CELLS

Cells were initially cultured submerged in equine airway culture media. Cells were plated either onto collagen pre-coated vented culture flasks (T25 or T75) for propagation prior to seeding or directly onto collagen pre-coated air-liquid-interface (ALI) transwell plates, as outlined in *Chapter 5*.

2.2.3.2b TRYPSIN TREATMENT TO SEPARATE CELLS

Trypsin-EDTA solution was used to collect adherent proliferated cells from culture flask or transwell insert surface. Any media in which the cells were submerged was

removed by suction and the cell surface washed with DPBS and then the washing was removed and discarded. The adherent cells were incubated with cell culture grade trypsin at 37 ^{o}C for 5 minutes or until cellular separation was microscopically apparent; at which point an equal volume of trypsin neutralizing solution (TNS) was added. The liquid was then remove from the flask and centrifuged (3000 rpm, 3 minutes) to pellet the cells; the supernatant was discarded and the cells re-suspended in fresh equine airway culture media ready for seeding.

2.2.3.2c AIR LIQUID INTERFACE CULTURE

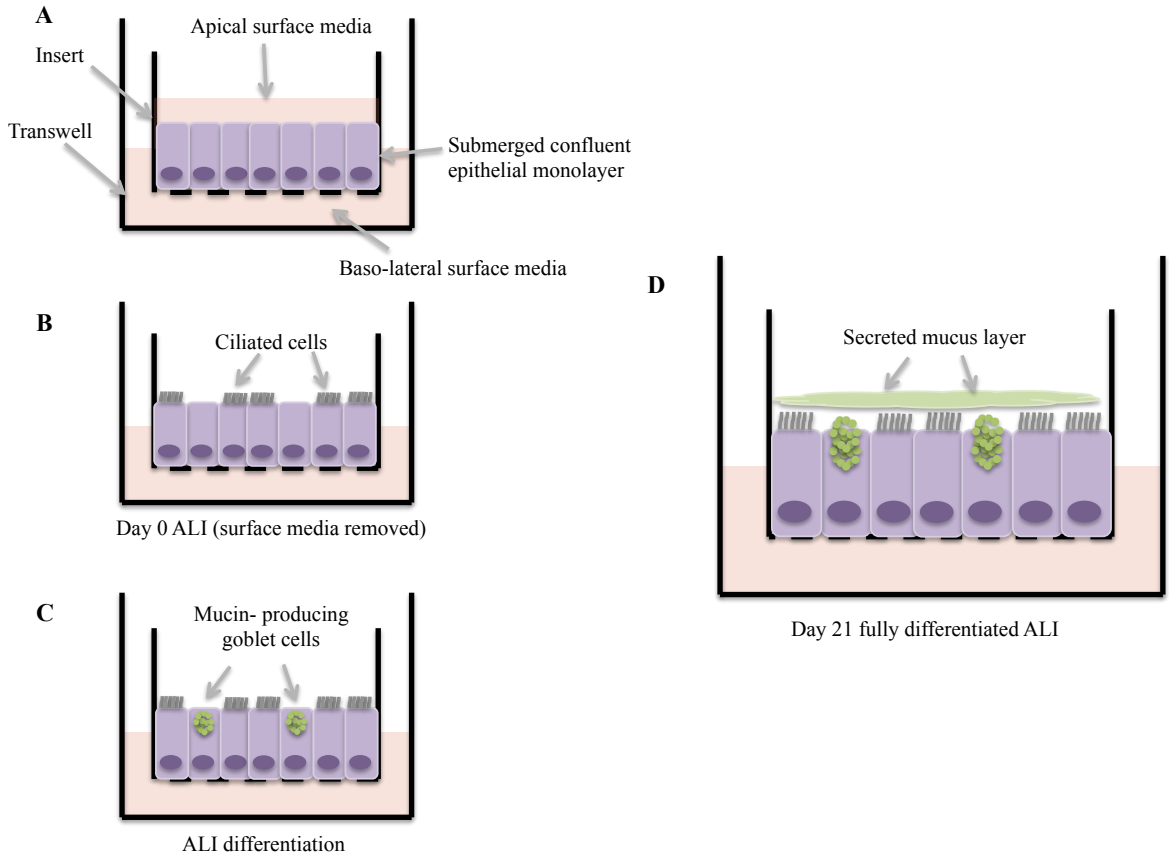
After propagation to approximately 90 % confluence (assessed by light microscopy), cells were cultured in an air-liquid interface system (ALI) as described previously (Fulcher *et al.*, 2005; Schwab *et al.*, 2010). The monolayer propagated cells were treated with trypsin-EDTA as described previously and seeded onto collagen pre-coated 12 mm diameter, 0.4 μm pore size, polyester transwell inserts in 12-well plates at an approximate concentration of 16.5 x 10^4 cells/ mL (*Fig. 2.7*).

Seeded cells were cultured submerged in equine airway cell culture differentiation media (containing retinoic acid) for 5 to 7 days with the same media basolaterally underneath the cells in the transwell. The apical surface media was changed once every 2-3 days, with fresh retinoic acid being added each time. The apical surface media was removed once a confluent monolayer of cells had been achieved, thus creating ALI culture (day 0). The basolateral media was then changed once every 2-3 days whilst the cells matured and differentiated. Average time to full differentiation from the start of ALI was 21 days (*Fig. 2.7*).

2.2.3.2d APICAL SURFACE WASHING TO REMOVE SECRETED MUCUS FROM ALI CELLS

The secreted mucus layer was removed from the apical surface of cells by application and gentle retrieval of 500 μL equine airway culture media (warmed to 37 ^{o}C). The collected mucus was stored at -20 ^{o}C for future analysis.

Fig. 2.7 **SCHEMATIC DIAGRAM OF AIR-LIQUID INTERFACE (ALI) CELL CULTURE. A**. Equine airway epithelial cells seeded onto porous transwell insert membrane proliferate to confluence submerged in equine airway culture medium. **B**. Once confluent the apical surface media is removed from the cell monolayer and cells are fed via the porous membrane insert on their baso-lateral side: ALI day 0. The cells start to differentiate with ciliated cells being produced first. **C**. As cell differentiation continues, mucin-producing goblet-type cells appear. **D**. At approximately day 21 the ALI culture is fully differentiated with mucus secreted on the apical surface. (Diagrams not to scale).



2.2.3.2e FROZEN STORAGE OF CELLS AND DEFROSTING

For freezing of freshly harvested epithelial cells, trypsin-separated proliferated primary culture or differentiated first or second passage cells for future research purposes; cells were re-suspended in 1 ml equine airway transport media containing serum and 10 % DMSO. Cells were frozen in 1 mL freezing vials slowly down to -80 ^{o}C using a Mister Frosty freezing apparatus and stored at -80 ^{o}C.

Cell vials removed from the -80 ^{o}C freezer were warmed at 37 ^{o}C for 3 minutes to defrost and then added to a vented T75 culture flask with 11 mL equine airway culture media and incubated as described above and allowed to proliferate. The media was changed after 24 hours to remove the DMSO.

CHAPTER 3: BIOCHEMICAL PROPERTIES OF MUCINS IN RAO HORSE AIRWAY MUCUS

3.1 INTRODUCTION

In the normal horse, the predominant polymeric equine airway mucins have been previously established to be Muc5b and Muc5ac (Gerber *et al.*, 2003; Rousseau *et al.*, 2011b; Rousseau *et al.*, 2007). It has also been established that in healthy Thoroughbred horses and those suffering from IAD that Muc5b is the predominant mucin, being more abundant than Muc5ac (Rousseau *et al.*, 2011b). In horses with RAO, gene expression for *Muc5ac* has been reported (Gerber *et al.*, 2003); however, the identity of secreted mucins and which one may predominate in mucus from airways of horses with RAO has yet to be established.

Horses suffering from RAO have increased tracheal mucus identifiable on endoscopy when they are symptomatic (during exposure) but also when they are asymptomatic (not-exposed) (Christley *et al.*, 2001; Jefcoat *et al.*, 2001; Robinson *et al.*, 2002; Robinson *et al.*, 2003). It is well established that the secreted, polymeric mucins are responsible for the viscous nature of mucus gels; however it is not yet known which mucins are the key components of this increased volume of mucus seen in RAO horses both whilst symptomatically affected and when their disease is clinically silent.

Human airway mucins from diseased subjects in which, as for RAO, increased airway luminal mucus is a common feature, have been demonstrated to differ in relative amounts and have different biochemical properties compared to their healthy counterparts in various diseases (Burgel *et al.*, 2007; Davies *et al.*, 1996; Kirkham *et al.*, 2002). It has been shown that although MUC5AC is the major airway mucin in health, MUC5B predominates in asthmatic airways (Groneberg *et al.*, 2002a; Sheehan *et al.*, 1999). In addition, the mucins in the mucus plugging the airways of a human asthma patient that died in *status astmaticus* was abnormal in size and morphology and had lower average charge density than mucins from healthy airways mucus (Sheehan *et al.*, 1999). We do not yet know whether RAO mucus mucin size, charge or charge density is different to or the same as those in the non-diseased horse.

The aim of this chapter of this thesis is to establish what mucins are present in the airway mucus of horses with RAO, which mucin if any predominates, and what are their biochemical properties. We know that Muc5b predominates in healthy horse airways, but we need to establish if this is also the case in the RAO diseased horse. Understanding if

there is a difference in secreted mucin composition of mucus in exposed and not-exposed horses and RAO-diseased horses and healthy control counterparts will also help to understand the pathology of the disease and whether therapeutic targets can be designed in the future. The following aims will help to determine if the hypothesis is true that Muc5b and to a lesser extent, Muc5ac are the major secreted polymeric mucins in RAO horse airway mucus, and differ in their amount, but not biochemically in terms of size and charge density, to healthy horse airway mucins:

- Aim 1: Determine the major airway mucins in horses with RAO, and which, if any, predominates.
- Aim 2: Determine if there is a difference in which mucins are present in mucus from exposed and not-exposed healthy and RAO horses.
- Aim 3: Determine the size distribution of RAO mucins and if they differ in size to mucins from healthy controls.
- Aim 4: Determine if RAO horse airway mucins have the same charge density and electrophoretic mobility as those of healthy horses.

3.2 RESULTS

In order to investigate the above hypothesis, airway mucus samples were collected and analysed from various sources. Mucus samples were first readily available from clinical cases of RAO and these were employed to establish which sampling technique was most suitable (tracheal wash, TW, or broncho-alveolar lavage, BAL) and to also identify which mucins were present in airway mucus of RAO horses. Subsequently, samples of airway mucus became available from a controlled experimental herd of horses, both RAO-diseased and healthy controls, and these were used to compare mucin content and properties between exposed and not-exposed animals. Mucus samples from all sources were excesses of samples collected for other diagnostic or scientific purposes unrelated to this project.

3.2.1 WHAT IS THE BEST SAMPLING TECHNIQUE TO COLLECT MUCUS FOR MUCIN ANALYSIS?

Airway mucus samples from one RAO diseased horse (RAO.CD.1; *Appendix I, Table AI.1*) were kindly provided for initial investigation of this question. The samples,

collected endoscopically via infusion and retrieval of sterile 0.9 % saline were performed at two separate anatomical locations in the airways of this horse: 2 samples were collected one month apart from the tracheal mucus pool by TW, one sample collected bronchial mucus via BAL at the same time point as the first TW. The samples were collected for clinical reasons by an independent veterinarian investigating the course of disease in an RAO horse patient. For TW samples, 100 mL of saline was injected and approximately 50 mL retrieved each time; whilst for BAL 300 mL saline was injected and approximately 50 mL retrieved. The sample size of each sample donated for this experimental investigation was 20 mL. Immediately after collection the samples were added to an equal volume (20 mL) of 8 M GuHCl. A further addition of 40 mL 4 M GuHCl was required to solubilize the two TW samples, whilst no further GuHCl was required for the BAL sample. 40 mL of each solubilized mucus sample (in 4 M GuHCl) was used to perform CsCl (starting density 1.4 g/ mL) density gradient purification; which was subsequently unloaded in 20 x 2 mL fractions from the bottom of the tubes (most dense fraction first). Analysis of fraction density, A_{280} (protein and nucleic acid) and PAS stain intensity on a slot blot (glycoproteins), identified the putative mucin-rich fractions were the more dense fractions 1 - 10 (1.38 - 1.61 g/ mL) of the gradients for the TW samples (*Fig. 3.1*). There was very little mucin detected in the BAL sample (*Fig. 3.1*). Fractions 1 - 10 from both TW samples were pooled separately and underwent a second CsCl density gradient (0.2 M GuHCl, starting density 1.5 g/ mL) purification, producing a further 20 x 2 mL fractions. Putative mucin-rich fractions from the second CsCl (fractions 1 - 10, density 1.5 - 1.7 g/ L) were identified by PAS staining and were pooled. The pooled samples were reduced (10 mM DTT) and alkylated (25 mM IAA), dialysed in water, freeze-dried and re-suspended before trypsin digestion in preparation for mass spectrometry.

The polymeric, gel-forming mucins present in the purified mucin-rich fractions from the TW samples were determined by tandem mass spectrometry of tryptic-peptides. Analysis of the mass spectrometry data confirmed the presence of both equine Muc5b (48 peptides in the first and 58 peptides in the second TW samples) and equine Muc5ac (47 peptides in first and 38 peptides in the second TW samples) (*Table 3.1*). Good sequence coverage for identified peptides present in the predicted polypeptide sequences N- and or C-termini and cys domains of Muc5b and Muc5ac was achieved from both samples (*Table. 3.1*). Matching peptides were not identified from the glycosylated domains of Muc5b or Muc5ac, as those large molecules were removed from the samples as part of sample preparation (refer to *Methods*). Many non-mucin peptides were identified in the mass-

spectrometry data analysis, including actin, keratin and trypsin peptides (data not shown). No mucin peptides were identified from the BAL sample because the fractions from the first CsCl purification were too dilute for a second purification step or mass spectrometry to yield any meaningful results. The two TW samples were collected 4 weeks apart from the same horse, during the course of treatment for RAO, and yet similar mucin sequence coverage is identified in both samples.

Fig. 3.1 **ANALYSIS OF GuHCl SOLUBILISED MUCUS SAMPLES COLLECTED BY TW OR BAL FROM A HORSE SUFFERING FROM RAO**. TW1 and TW2 are tracheal wash mucus samples collected from the same RAO horse 4 weeks apart. BAL is a broncho-alveolar lavage mucus sample collected from the same horse at the same time point as TW1. **A.** PAS stain of a slot blot of CsCl density gradient fractions from each mucus sample. 20 x 2 mL fractions were unloaded from the bottom of the tube (most dense fractions unloaded first). 100µL sample loaded per slot blot well; numbers refer to fractions. **B.** Density (black diamonds), Absorbance A_{280} (grey squares) and normalised PAS stain intensity (pink triangles) for fractions of the CsCl density gradient for each TW mucus sample (data for BAL sample not shown due to lack of positive response).

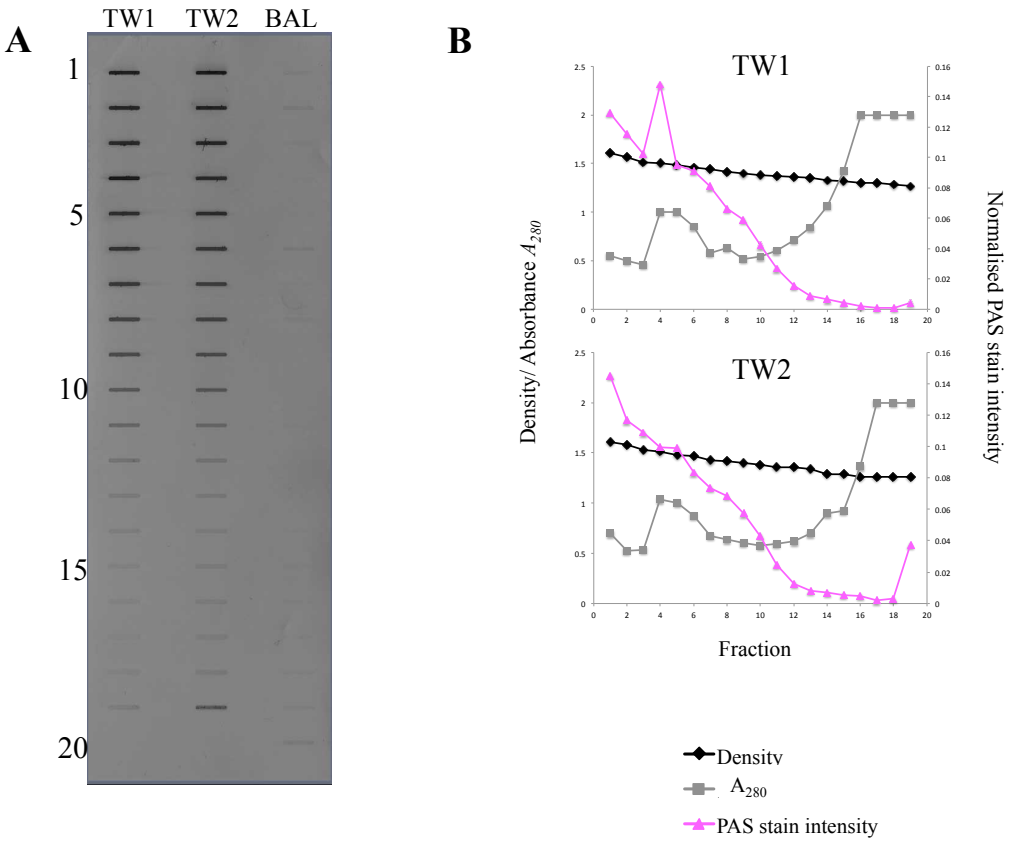
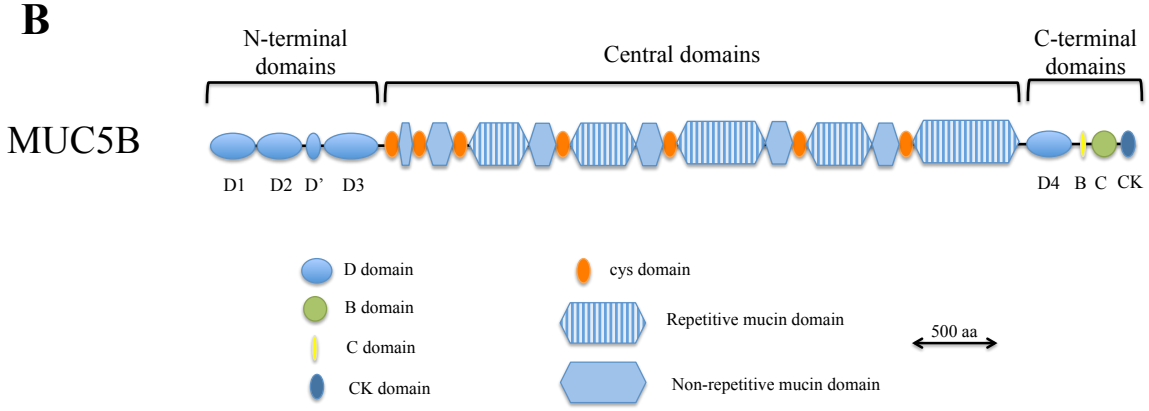


Table. 3.1 **MASS SPECTROMETRY ANALYSIS OF GuHCl SOLUBILISED MUCUS SAMPLES FROM A HORSE SUFFERING FROM RAO**. TW1 and TW2 are tracheal wash mucus samples collected from the same horse 4 weeks apart. The mucus samples underwent two CsCl density gradient purifications; putative mucin-rich fractions were pooled, reduced (10 mM DTT) and alkylated (25 mM IAA), dialysed in water, freeze-dried and re-suspended before trypsin digestion and tandem mass spectrometry of the tryptic-peptides. **A**. Mass spectrometry sequence coverage for predicted equine airway mucins (number of identified peptides and percentage of sequence covered) for TW1 and TW2 mucus samples are shown. The BAL sample was too dilute for mucin peptide identification. B. Schematic diagram for reference representing equine Muc5b orthologue (human polymeric secreted mucin MUC5B) structural domains with von Willebrand factor (vWF) like N- and C- terminal domains (B, C, CK and D domains), and a representation of the central region containing multiple cys and mucin domains (MD). Figure adapted from Thornton *et. al*., (2008). aa denotes amino acid.

A

Predicted Mucin Sequences	TW1 Sequence Coverage	TW2 Sequence Coverage
Equine Muc5b prediction with N terminus	**34 peptides (22%)**	**37 peptides (26%)**
Equine Muc5ac prediction (N and C termini and Cys domains)	**47 peptides (11%)**	**38 peptides (10%)**
Equine Muc5b Cys domain potential	**11 peptides (18%)**	**12 peptides (21%)**
Equine Muc5b Cys domain 1 potential	-	**6 peptides (20%)**
Equine Muc5b Cys domain 2 potential	**12 peptides (34%)**	**13 peptides (44%)**

B

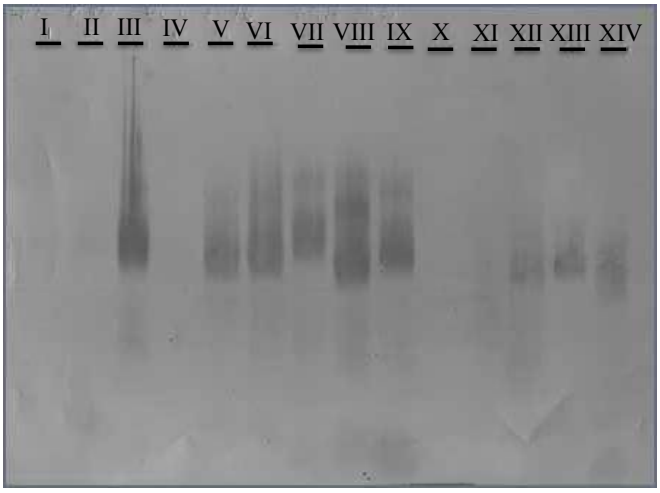


The methodology employed using two CsCl density gradient steps is a useful way to purify mucins for further biochemical analysis; however this process can be quite time consuming. Agarose gel electrophoresis and Western blotting with immunodetection can be used to profile mucins present in mucus samples without prior purification (Kirkham *et al.*, 2002; Rousseau *et al.*, 2011b). From the analysis performed above, it seemed that the BAL sample was too dilute for mucin detection using the purification methods but it was worth investigating if this was also the case for unpurified mucus samples before ruling BAL out as a sampling method for mucin analysis. Agarose gel electrophoresis works on charge density of the mucins (mainly due to negative charges on the glycans), allowing comparison of mucins across a range of horses. We were kindly provided with airway mucus samples from 14 different clinical cases of RAO from a veterinary clinic (*Appendix I: Table AI.2*). The samples were excesses not used by the clinic; the samples had been collected from different clinical cases and by different referring veterinarians. We were provided with 2 mL of sample from each horse, solubilised in 2 mL of 8 M GuHCl (information on original volume of saline infused and retrieved was not available). Two samples were BAL samples, the remainder were TW samples. Although these samples were from varying sources and therefore not controlled in any way, we decided it would nevertheless be useful to use the samples for agarose gel electrophoresis to compare mucin charge density across a range of horses suffering from the disease we are interested in, and also to confirm if BAL samples were unsuitable for our methodologies of mucin analysis.

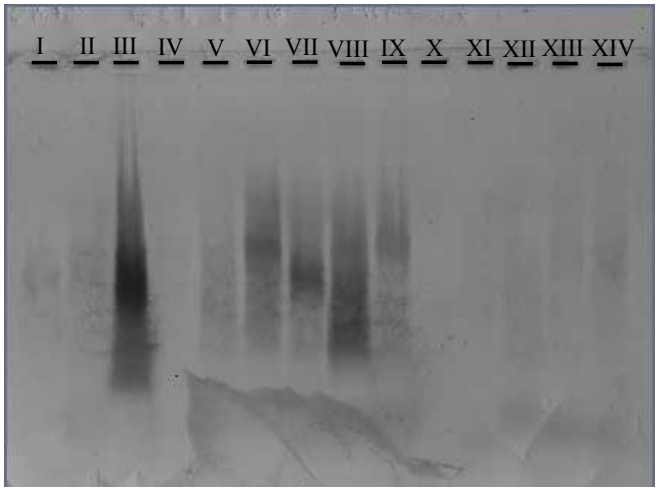
Having identified Muc5b and Muc5ac as mucins present in the first RAO horse sample, the 14 unpurified RAO equine patient airway mucus samples were used to perform agarose gel electrophoresis followed by Western blotting with immunodetection for these two mucins (*Fig. 3.2*). Horses were arbitrarily allocated Roman numeral identifiers I - XIV. The two BAL samples were from horses I and XI, while the remaining 12 samples were TW samples. The GuHCl solubilized samples were reduced (10 mM DTT) and alkylated (25 mM iodoacetamide) and dialysed in 6 M urea prior to agarose gel electrophoresis. The stain intensity for both mucins varied between horses, with very little Muc5b or Muc5ac mucin detection for 5 / 14 samples, included in which were both BAL samples. On the gels, Muc5ac migrates on average further than Muc5b. There is evidence of two distinct migratory bands for Muc5b, the faster of which appeared to have a similar mobility between samples from different horses. The electrophoretic mobility of Muc5ac appeared to be more varied between samples from different horses, compared to Muc5b.

Fig. 3.2 **AGAROSE GEL ELECTROPHORESIS OF MUC5B AND MUC5AC MUCINS IN TW AND BAL MUCUS SAMPLES FROM HORSES SUFFERING FROM RAO**. Roman numerals refer to individual horses, of which I and XI were BAL samples, an all other samples were TW samples. GuHCl solubilised mucus samples (original sample volume 2 mL with 2 mL 8 M GuHCl added) were reduced and alkylated, dialysed into 6 M urea and then subjected to agarose gel electrophoresis (150 µL sample/ well; 30 V, 16.5 hours). After electrophoresis, mucins were transferred to nitrocellulose membrane by vacuum transfer and mucins detected with **A.** eqMAN5b-1 (equine Muc5b) **B.** eqMAN5ac-1 (equine Muc5ac) using AP-conjugated secondary antibody and NBT and BCIP substrates.

A



B



Taking into account the data from density gradient centrifugation, mass spectrometry analysis and agarose gel electrophoresis and Western blotting of a variety of mucus samples from RAO horse airways, we have established that Muc5b and Muc5ac are the predominant mucins in RAO airway mucus and that mucins in BAL fluid are more than likely too dilute for use in the methodologies currently employed to study mucins. Mass spectrometry detection of mucins from BAL samples was not feasible due to the dilute nature of these samples; however if BAL samples had first been concentrated in a 100 kDa MWCO spin column (refer to *Methods*), then it may have been possible to identify mucins from these samples. For all subsequent experiments, TW samples only were used.

These initial experiments were useful for establishing the preliminary data; however they were not well controlled and there was therefore inevitably a large variation between samples. The next samples that became available were from horses in an experimental herd, and as such, sampling techniques and the environmental conditions that the horses were exposed to could be more rigorously controlled, limiting variables between samples. These samples were kindly provided by The University of Montreal.

As discussed previously (refer to *introduction*), the clinical signs of RAO become apparent when a horse is managed in a trigger environment. A typical trigger, or "exposed" environment for most RAO horses would be dusty, poor-ventilated indoor housing with straw bedding and feeding of mouldy hay; whilst clinical signs usually resolve, with the horse becoming asymptomatic in a "not-exposed" outdoors open airspace with removal of mouldy feedstuffs. As mucus hypersecretion is a key part of disease pathology, and yet RAO horses also have higher visible amounts of tracheal mucus even when clinical signs resolve, it is important to investigate whether mucins, the structural components of mucus, differ between these exposed and not-exposed states.

3.2.2 ANALYSIS OF MUCINS IN MUCUS SAMPLES FROM SYMPTOMATIC AND ASYMPTOMATIC RAO HORSES

Transendoscopic TW mucus samples were collected from an experimental herd of RAO horses (n = 10) managed under 2 separate environmental conditions (*Appendix I: Table AI.3*). The samples were excesses of fluid collected as part of unrelated experiments for which ethical consent had been granted under the University of Montreal guidelines. Sampling was performed from an asymptomatic unexposed set of RAO horses, managed in a clean air outdoor environment (n = 4; horses 1 - 4), and from a separate set of

symptomatic exposed horses (after at least 14 days' management in a dusty stable environment being fed dry mouldy hay) (n = 6; horses 5 - 10). At the time of sample biochemical processing, the operator was blinded to the identification of the horses to avoid bias, and only subsequently have their identities been revealed and the numbering identification 1-10 been assigned. Signalment and historical lung function data were not provided until after data collection (refer to *Appendix I: Table AI.3, Fig. AI.3*).

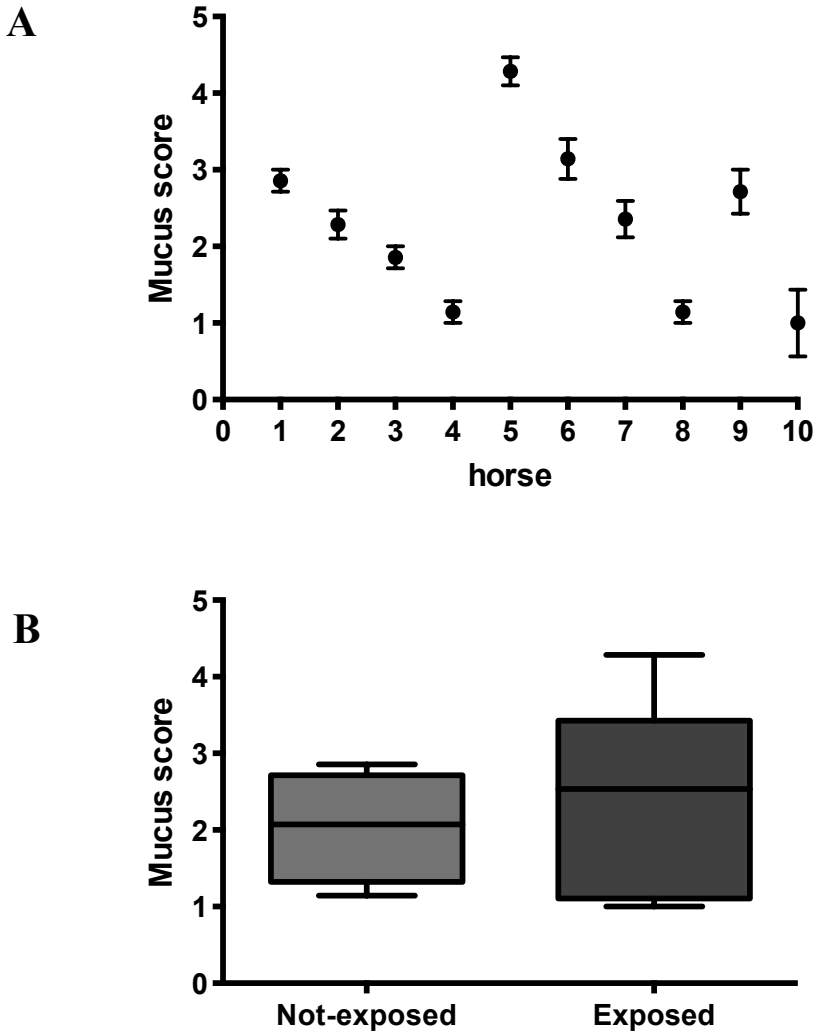
3.2.2.1 TRACHEAL MUCUS SCORE AND LUNG FUNCTION DATA

Lung function testing on these horses was previously carried out by our collaborators, Dr. J.P. Lavoie and colleagues (University of Montreal), in earlier experiments not related to the current study. The horses all historically had a measurable change in lung function, as measured by maximal change in intra-pleural pressure (termed Delta P), between not-exposed and exposed states, consistent with a diagnosis of RAO (refer to *Appendix I: Fig. AI.3*). Video-endoscopic footage of tracheal mucus was available from the time of mucus sampling and this was used to assign an endotracheal mucus accumulation score to each horse for the time of mucus sampling. Seven independent observers blinded to the identity of the horses were asked to examine the footage and score the mucus accumulation according to the published scale by Gerber *et al.* (2004). The exposed group of horses did not have a significantly different endoscopic tracheal mucus accumulation score at the time of mucus sampling to the not-exposed group of horses (Mann-Whitney U, P = 0.6476) (*Fig. 3.3*).

Tracheal mucus was collected from the horses trans-endscopically via infusion of 100 mL sterile 0.9 % saline with suction retrieval. The volume of mucus retrieved was significantly less than that injected and varied in volume between horses (refer to *Appendix I: Table AI.6*). Following mucus collection, all samples were fully solubilised by adding an equal volume of 8 M GuHCl to the retrieved sample volume (which varied between individual horses from 7.5 to 18 mL, refer to *Appendix I: Table AI.6*). Due to variation in sample volume retrieved, mucins in the samples were concentrated on a 100 KDa MWCO spin column.

We wanted to investigate if the electrophoretic mobility (related to charge density) as well as the size, of the major polymeric airway mucins Muc5b and Muc5ac, varied between exposed and not-exposed RAO horse mucus samples and therefore the following experiments were performed.

Fig. 3.3 **ENDOSCOPIC TRACHEAL MUCUS ACCUMULATION SCORE FROM RAO DISEASED EXPERIMENTAL HERD HORSES 1-10**. Endoscopic tracheal mucus accumulation score at time of mucus sampling was assessed retrospectively by review of recorded endoscopic footage, by 7 independent observers blinded to horse identity using the mucus accumulation score published by Gerber et. al. (2004). **A.** individual horse scores (mean +/- SEM). **B**. Grouped "exposed" and "not-exposed" RAO horses: no significant difference in mucus accumulation score between groups, Mann-Whitney U, P = 0.6476 (Whiskers = min and max).



3.2.2.1a VARIATION OF MUCIN MUC5B AND MUC5AC DETECTED ON AGAROSE GEL ELECTROPHORESIS BETWEEN HORSES IS NOT RELATED TO EXPOSURE STATUS

An aliquot of the GuHCl-solubilised concentrated TW mucus samples (5 mL from each sample) was reduced and alkylated and then buffer exchanged for 6 M urea using a 100 KDa MWCO spin column in preparation for agarose gel electrophoresis, Western blotting and immunodetection with antisera specific for equine Muc5b and Muc5ac (*Fig. 3.4*). There was a variation of mucin detection between diseased horses, whether sampled either during not-exposed or exposed environmental conditions for both mucins Muc5b and Muc5ac (*Fig. 3.4*). A faster migrating species of Muc5b was identified in horses 2 and 4, who also had the strongest bands for Muc5b, and both of which were not-exposed horses (*Fig. 3.4*). Horses 2 (asymptomatic not-exposed group) and 7 (symptomatic exposed group) had the strongest bands for Muc5ac. Horse 3 (not-exposed group) had barely detectable Muc5ac and a comparatively light band for Muc5b uptake compared to the other horses.

We found an interhorse-variation in mucin-positive stain on the agarose blots, and also that there was a wide variety of transendscopic tracheal mucus score at time of sampling, so we were interested to see if there was a correlation between these two findings. There does not appear to be any correlation (Pearson's correlation) for either Muc5b ($r = -0.3415$, $P = 0.3341$) or Muc5ac ($r = -0.1712$, $P = 0.6363$) stain intensities with tracheal mucus score (*Fig. 3.5*). The stain intensity could be related to the volume of sample retrieved, which also varied between animals. We investigated this and found no correlation of the volume of sample retrieved with the agarose stain intensity for Muc5b ($r = 0.107$, $P = 0.6382$) or Muc5ac ($r = -0.1052$, $P = 0.7723$) (*Fig. 3.5*). We then investigated and found no correlation between the volume of the sample retrieved and the mucus accumulation score for the time of mucus sampling ($r = -0.06148$, $P = 0.8660$) (*Fig. 3.5*).

The lack of correlation between the tracheal mucus accumulation score and the volume of sample retrieved indicates that the sampling technique employed is probably not achieving a representative sample of the mucus that can be visualised in the trachea. Despite the mucus sample not being representative of the mucus visualized in the trachea, we would still have expected the retrieved sample volume to have potentially correlated with the mucin stain intensity on the gel blots. The lack of correlation of mucin content with volume retrieved perhaps indicates that the mucin content of mucus is not related to the amount of mucus retrieved by the methods employed here.

Fig. 3.4 **AGAROSE GEL ELECTROPHORESIS OF MUCUS SAMPLES FROM RAO HORSES IN AN EXPERIMENTAL HERD.** Numbers 1 - 10 refer to individual RAO horses, of which horses 1 – 4 were asymptomatic at the time of TW sampling, whilst horses 5 - 10 were symptomatic, having been exposed to a challenge environment for at least 2 weeks prior to TW sampling. Horse identity was blinded at time of sample processing hence random number order. 4 M GuHCl solubilised samples were reduced and alkylated, and then buffer exchanged for 6 M urea using a 100 KDa MWCO spin column before being subjected to agarose gel electrophoresis (150 µL sample/ well; 30 V, 16.5 hours). After electrophoresis, mucins were transferred to nitrocellulose membrane by vacuum transfer and mucins detected with **A.** eqMAN5b-1 (equine Muc5b) **B.** eqMAN5ac-1 (equine Muc5ac) using AP-conjugated secondary antibody and NBT and BCIP substrates. Black solid arrows represent the migration position of the equine major Muc5b and Muc5ac bands; red arrow indicates position of faster migrating species of Muc5b in horses 2 and 4. Red bracket indicates position of variably faster migrating species of Muc5ac. **C** Densitometry stain intensity corresponding to major migratory band of mucins Muc5b and Muc5ac on gel blots.

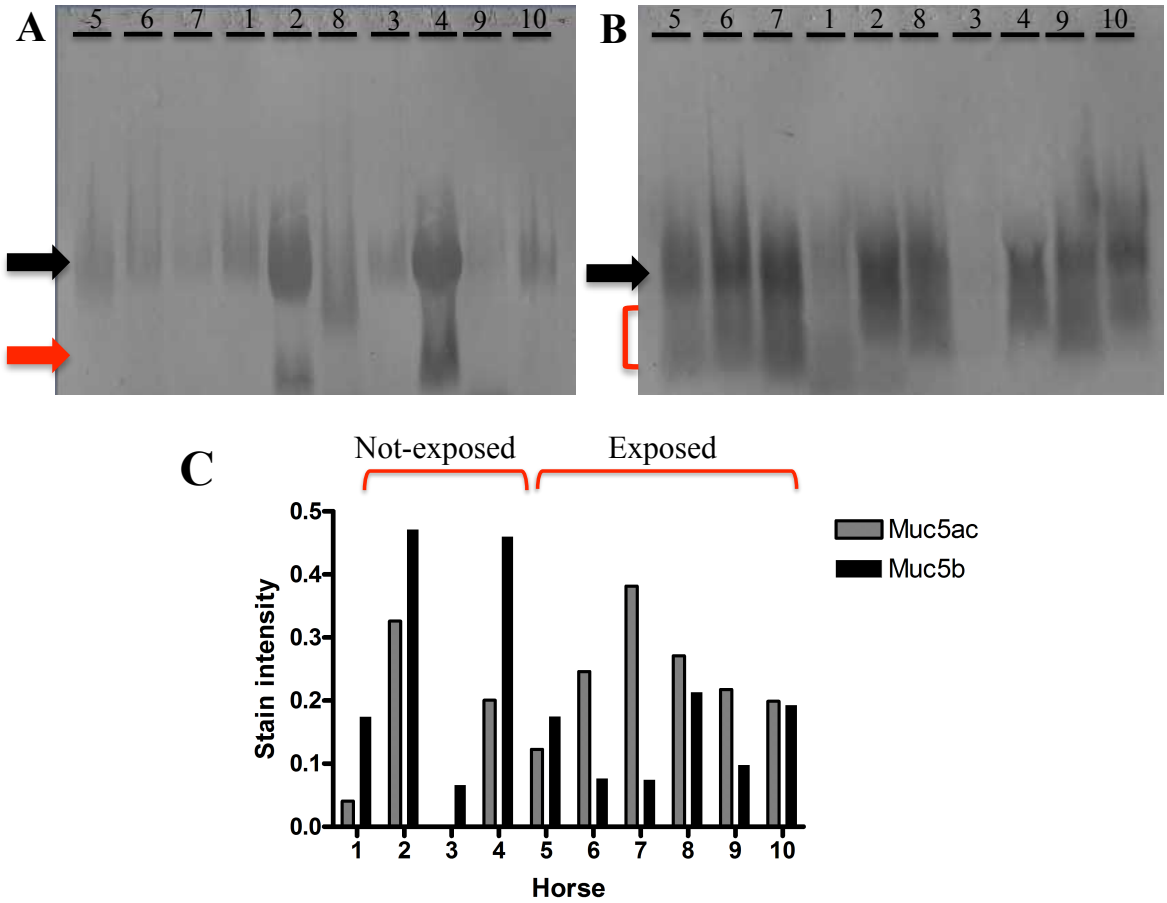
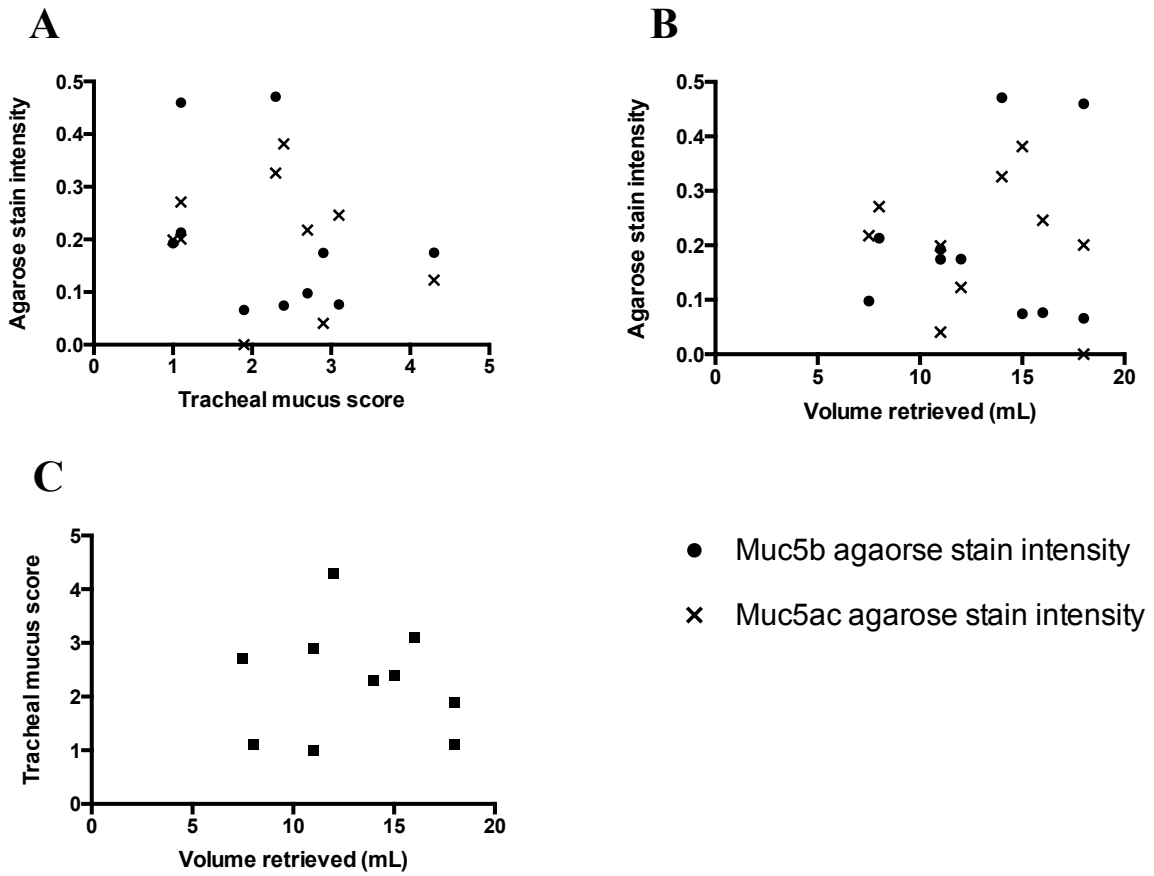


Fig. 3.5 **CORRELATION OF TRANS-ENDOSCOPIC TRACHEAL MUCUS ACCUMULATION SCORE AT THE TIME OF MUCUS SAMPLE COLLECTION WITH MUCIN MAJOR MIGRATORY BAND STAIN INTENSITY ON AGAROSE GEL ELECTROPHORESIS BLOT** from *Fig. 3.4*. (Pearson's correlation). **A.** Agarose gel electrophoresis blot stain intensity correlation with tracheal mucus accumulation score (for Muc5b r = -0.3415, P = 0.3341; for Muc5ac r = -0.1712, P = 0.6363). **B**. Agarose gel electrophoresis blot stain intensity correlation with volume of sample retrieved (for Muc5b r = 0.107, P = 0.6382; for Muc5ac r = -0.1052, P = 0.7723). **C**. Correlation of tracheal mucus accumulation score with volume of mucus sample retrieved (r = -0.06148, P = 0.8660).



We were limited in what we could alter about the caveats of the experiments discovered so far, in that we had been provided with the mucus samples collected in a particular way, and, despite the inadequacies of mucus sample collection, that was all we

had available in order to analyse the mucins within those mucus samples. So, we decided to continue to investigate at least the size distribution and mass spectra data from the samples, as these would still give us important information regarding the mucins in the samples even if the differences between individual horses could not be related to either the amount of mucus visualised in the trachea or the amount of sample retrieved.

3.2.2.2 ANALYSIS OF THE SIZE DISTRIBUTION OF THE MUCINS

One aim of this chapter was to determine if RAO mucins are polydisperse in size, which can be investigated by examining distribution of mucins across fractions of varying density collected by rate zonal centrifugation on a pre-formed gradient. Rate zonal centrifugation of GuHCl solubilised airway mucus samples from the asymptomatic not-exposed (1 - 4) and symptomatic exposed (5 - 10) RAO horses were performed and the fractions of increasing density were slot blotted and probed for mucin presence using PAS and immunodetection for equine Muc5b and Muc5b stains (*Fig. 3.6* and *Fig. 3.7*). Mucin detection across the range of fractions for each individual horse confirmed the polydisperse size nature of the equine airway mucins Muc5b and Muc5ac. There was no significant difference between individual horse mucin stain intensity distribution across fractions ($P > 0.5$, Kruskal-Wallis) for PAS or either mucin stain (*Fig 3.6*). There was no significant difference in PAS, Muc5b or Muc5ac stain intensity distribution across the fractions ($P > 0.05$; Kruskal-Wallis test) between individual horses in each group (comparing all not-exposed horses against each other and comparing all exposed horses against each other, data not shown). There was no significant difference ($P > 0.5$, Mann-Whitney U) in stain intensity distribution for all three stain types between exposed and not-exposed groups of horses (*Fig. 3.7*). It would seem that there is no change in mucin size distribution between exposed and not-exposed RAO horses, just that the amounts of mucus and mucins varies between individuals. However, the numbers of horses in each group ($n = 4$ asymptomatic and $n = 6$ symptomatic respectively) is low and therefore a caveat of these experiments is that the low numbers may not have enough power to show significant differences.

Fig. 3.6 **ANALYSIS OF THE SIZE DISTRIBUTION OF THE MUCINS.** GuHCl solubilised airway mucus samples from horses suffering from RAO were loaded onto a preformed GuHCl gradient and centrifuged in a Beckman SW40 swing-out rota at 4 ^{0}C 40,000 rpm for 2.5 hours. 24 x 500 µL fractions of increasing density were collected (molarity calculated from measured refractive index of each fraction). Slot blot and detection using PAS and immunodetection for equine Muc5b and Muc5b were performed for each fraction. Graphs show stain intensity for individual horses, identified by numbers: asymptomatic RAO horses 1 - 4 (not exposed to a challenge environment); symptomatic RAO horses 5 - 10 exposed to a challenge environment.

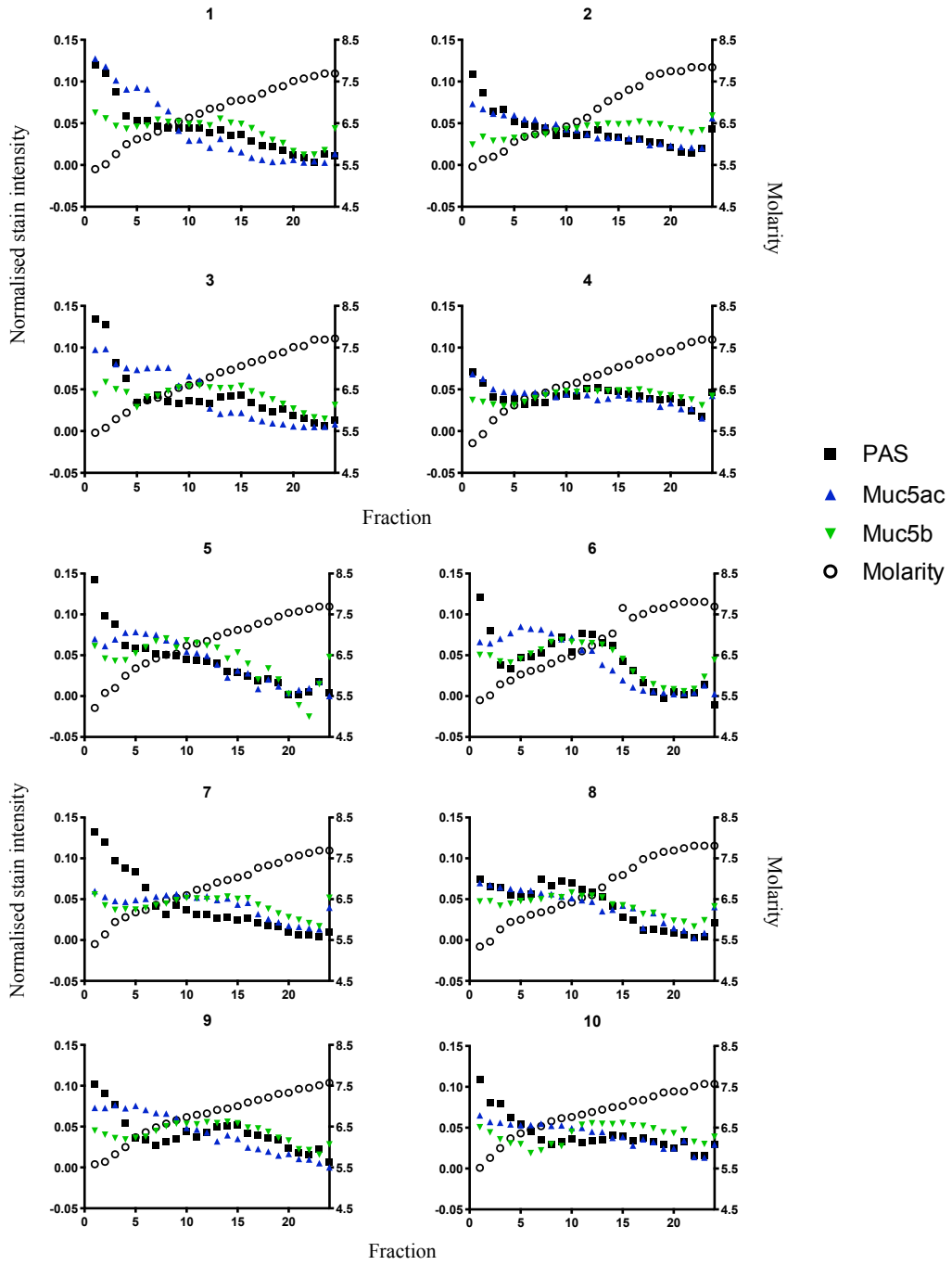
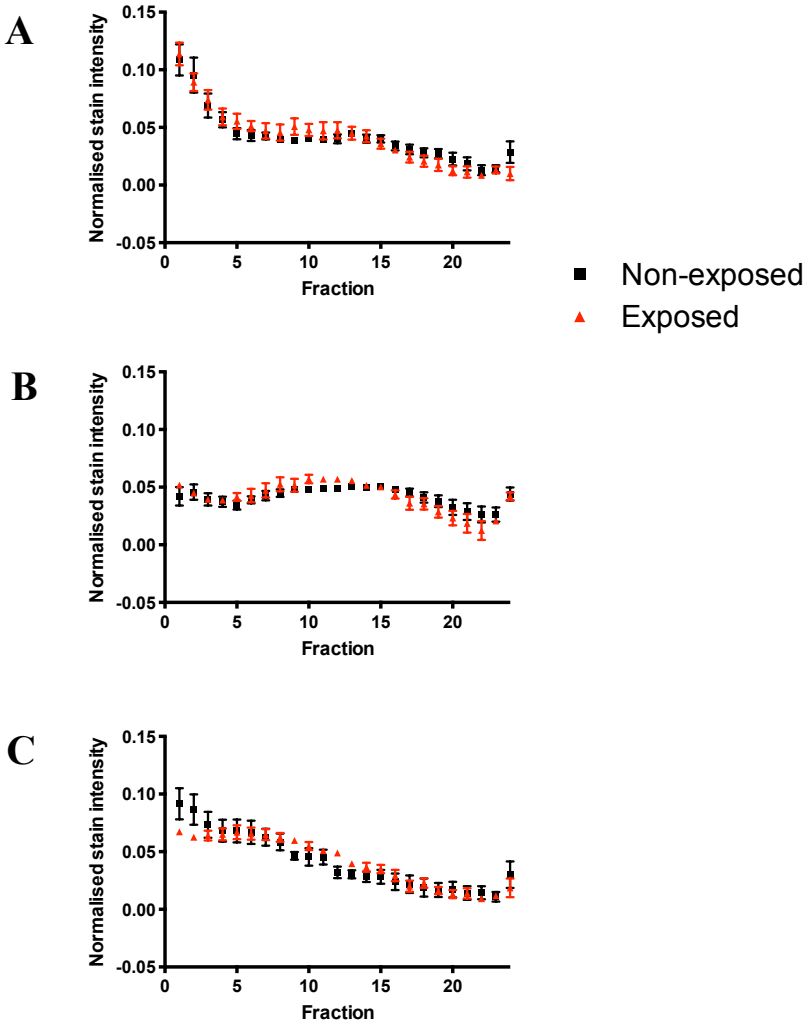


Fig. 3.7 **SIZE DISTRIBUTION OF MUCINS FROM SYMPTOMATIC AND ASYMPTOMATIC RAO HORSES: SEPARATION BY RATE ZONAL CENTRIFUGATION.** GuHCl solubilised airway mucus samples from horses suffering from RAO were loaded onto a preformed GuHCl gradient and centrifuged in a Beckman SW40 swing-out rota at 4 ^{0}C 40,000 rpm for 2.5 hours. 24 fractions of increasing density were collected. Slot blot and detection using PAS and immunodetection for equine Muc5b and Muc5ac were performed for each fraction. Graphs show mean (+/- SEM) stain intensity for fractions from horses grouped into exposed (red triangle) and not-exposed (black square) groups. **A**. PAS**. B**. Muc5b **C**. Muc5ac. No significant difference (P > 0.5, Mann-Whitney U) for all 3 stain types between exposed and not-exposed groups of horses.



3.2.2.3 MASS SPECTROMETRY

Airway mucus samples collected from the asymptomatic not-exposed and symptomatic exposed RAO-diseased horses 1-10 were solubilized in GuHCl, reduced and

alkylated, purified by centrifugation on a 100 KDa molecular weight cut-off column and trypsin digested before protein identification by tandem mass spectrometry (*Table 3.2*). Peptides from Muc5b were identified in the samples from all horses except horse 3 (asymptomatic) and horse 9 (symptomatic). Equine Muc5ac peptides were identified in not-exposed horses 2 and 4, and in mucus from exposed horses 6,7 and 10. No peptides from other polymeric airway mucins were identified. No equine Muc5b or Muc5ac peptides were identified in the samples from horses 3 (asymptomatic) and 9 (symptomatic). This correlates with the data from the agarose gel electrophoresis for horse 3 but not for horse 9. Muc5ac is identified on the agarose blots but not in the MS data for horses 2, 5, 8 and 9. Muc5b is identified on the agarose gel but but not in the MS data for horse 9 only. The discrepancies identified here in the identification of Muc5b and Muc5ac in the agarose gel blots versus the MS data may be due to a number of factors. Muc5b is identified more frequently in the MS data, whilst the stain intensity for Muc5ac is stronger on the gel blots. The eqMAN5ac-1 antibody has been shown to be far more sensitive than the eqMAN5b-1 antibody (Rousseau *et al.*, 2011b), hence a far smaller amount of Muc5ac is needed to be present in a mucus sample for it to be identified compared to Muc5b. Mucin may be lost from samples during their processing for MS and thus if Muc5b is actually the more abundant mucin, this is reflected in the MS data which identifies it more readily than Muc5ac.

The data gathered from the above experiments confirmed that Muc5b, and to a lesser extent Muc5ac, are the predominant polymeric mucins in airway mucus from horses suffering from RAO. It also confirmed that the mucins are polydisperse and there is no significant size difference of the mucins in mucus samples from RAO horses when they are clinically silent (not-exposed) or showing signs of disease (exposed). For the next experiments comparing mucins in mucus samples from RAO horses and healthy controls in exposed and not-exposed environments, we now know to concentrate on Muc5b and Muc5ac. A caveat of the preceding experiments is that the exposed and not-exposed horses were different individuals; therefore we wanted to address this in the following experiments, being able to compare not-exposed to exposed mucus samples form the same individuals, and also to use samples from healthy control animals that were not sufferers of RAO.

Table 3.2 **MASS SPECTROMETRY OF RAO HORSE AIRWAY MUCUS SAMPLES.** Airway mucus samples collected from the asymptomatic not-exposed (1 - 4) and symptomatic exposed (5 - 10) RAO-diseased horses were solubilized in GuHCl, reduced and alkylated, concentrated by centrifugation on a 100 KDa molecular weight cut-off filter column and trypsin digested before protein identification by tandem mass spectrometry. Sequence coverage for predicted equine airway mucins is shown (number of identified peptides and percentage of sequence covered).

		Number of identified peptides (% sequence coverage)			
Predicted mucin sequences		eqMuc5b Cys domain	eqMuc5b Cys domain 2	eqMuc5b N terminus	eqMuc5ac N & C termini & cys domains
Not-exposed RAO horses	**Horse 1**	7 (12%)	8 (21%)	19 (11%)	10 (2%)
	Horse 2	-	8 (23%)	24 (14%)	-
	Horse 3	-	-	-	-
	Horse 4	7 (12%)	10 (23%)	32 (18%)	9 (2%)
Exposed RAO horses	**Horse 5**	10 (17%)	7 (14%)	7 (4%)	-
	Horse 6	8 (12%)	-	13 (7%)	4 (1%)
	Horse 7	4 (9%)	5 (16%)	16 (8%)	23 (5%)
	Horse 8	-	-	5 (3%)	-
	Horse 9	-	-	-	-
	Horse 10	5 (8%)	7 (24%)	-	12 (8%)

3.2.3 MUCUS SAMPLES FROM RAO DISEASED HORSES AND HEALTHY CONTROLS (11 - 16 = control, 17 - 22 = RAO)

Transendoscopic TW mucus samples were collected from control horses (n = 6; horses 11 - 16) and RAO horses (n = 6; horses 17 - 22) managed under identical environmental and feeding conditions (*Appendix I: Table AI.4*). Sampling was performed first when the horses were managed in a clean air (not-exposed) environment (with the RAO-diseased horses asymptomatic) and then after 30 days' management in a dusty stable environment, bedded on straw and fed mouldy hay, making the RAO-affected horses

symptomatic (exposed environment). In this way each horses acted as its own not-exposed control to compare to the exposed environmental conditions.

3.2.3.1 COMPARISON OF TRACHEAL MUCUS ACCUMULATION SCORE BETWEEN HORSES AND EXPOSURE STATES

Lung function testing on these horses was carried out by our collaborators, Dr. J.P. Lavoie and colleagues (University of Montreal). The RAO horses all had a measurable change in lung function, as determined by a maximal change in intrapleural pressure (termed Delta P), between not-exposed and exposed states, consistent with a diagnosis of RAO; whilst the control horses do not have a significant change in lung function between exposure states (refer to *Appendix I: Fig. AI.4*). Video-endoscopy was used during the mucus collection procedure to enable endoscopic scoring of mucus accumulation. Seven observers each scored tracheal mucus accumulation on two separate occasions, one month apart; giving a total of 14 observations per mucus sampling time per horse. Observers were blinded to the identity of each horse. Mean tracheal mucus score for each horse when not-exposed and exposed can be seen in *Fig. 3.9*. There was a significant difference in mucus accumulation score between control and RAO horses, with diseased horses having a significantly increased tracheal mucus score compared to healthy control horses, during both not-exposed and exposed conditions (P = 0.0043, Mann-Whitney U) (*Fig. 3.8*). There was no significant difference in tracheal mucus score between control horses when not-exposed or exposed (P = 0.8438, Wilcoxin signed rank) or for the RAO horses between exposed and not-exposed states (P =0.0938, Wilcoxin signed rank) (*Fig. 3.8*).

Fig. 3.8 **TRACHEAL MUCUS ACCUMULATION SCORE OF EXPOSED AND NOT-EXPOSED RAO AND HEALTHY CONTROL HORSES.** Video-endoscopy was used during mucus collection to enable endoscopic scoring of tracheal mucus accumulation. Seven observers each scored video footage of tracheal mucus accumulation on two separate occasions, one month apart; giving a total of 14 observations per mucus sampling time per horse. Observers were blinded to the identity of each horse. **A**. Mean tracheal mucus accumulation score (+ SEM) for each individual horse before (not-exposed) and after exposure to an environment that induces clinical signs of disease in RAO-affected horses. Numbers are identifiers for each individual horse where 11 - 16 are control animals and 17 - 22 are RAO-diseased horses. **B**. trans-endoscopic mucus accumulation scores of groups of horses (control vs RAO horses, exposed (E) vs not-exposed (NE). Mann-Whitney U, **P = 0.0043. (Refer to *Appendix AI.8* for raw data).

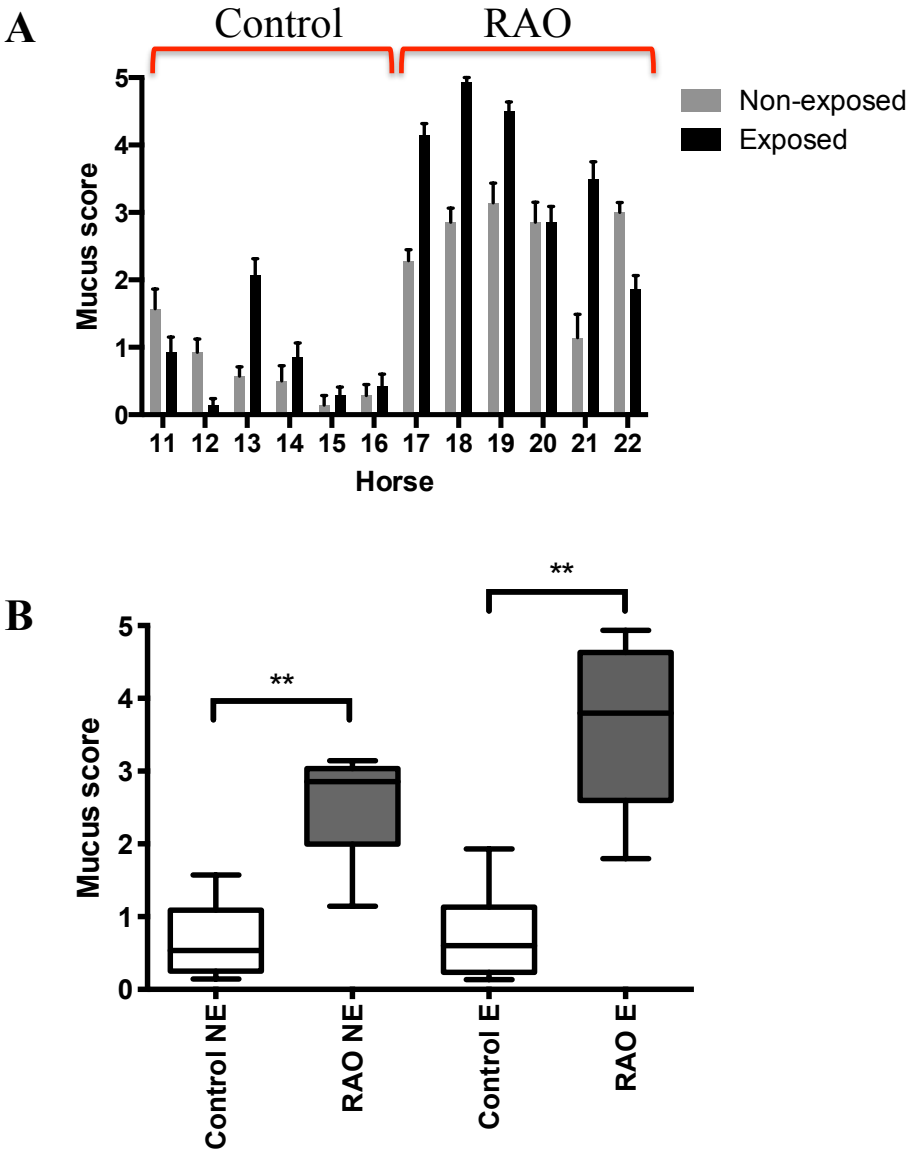
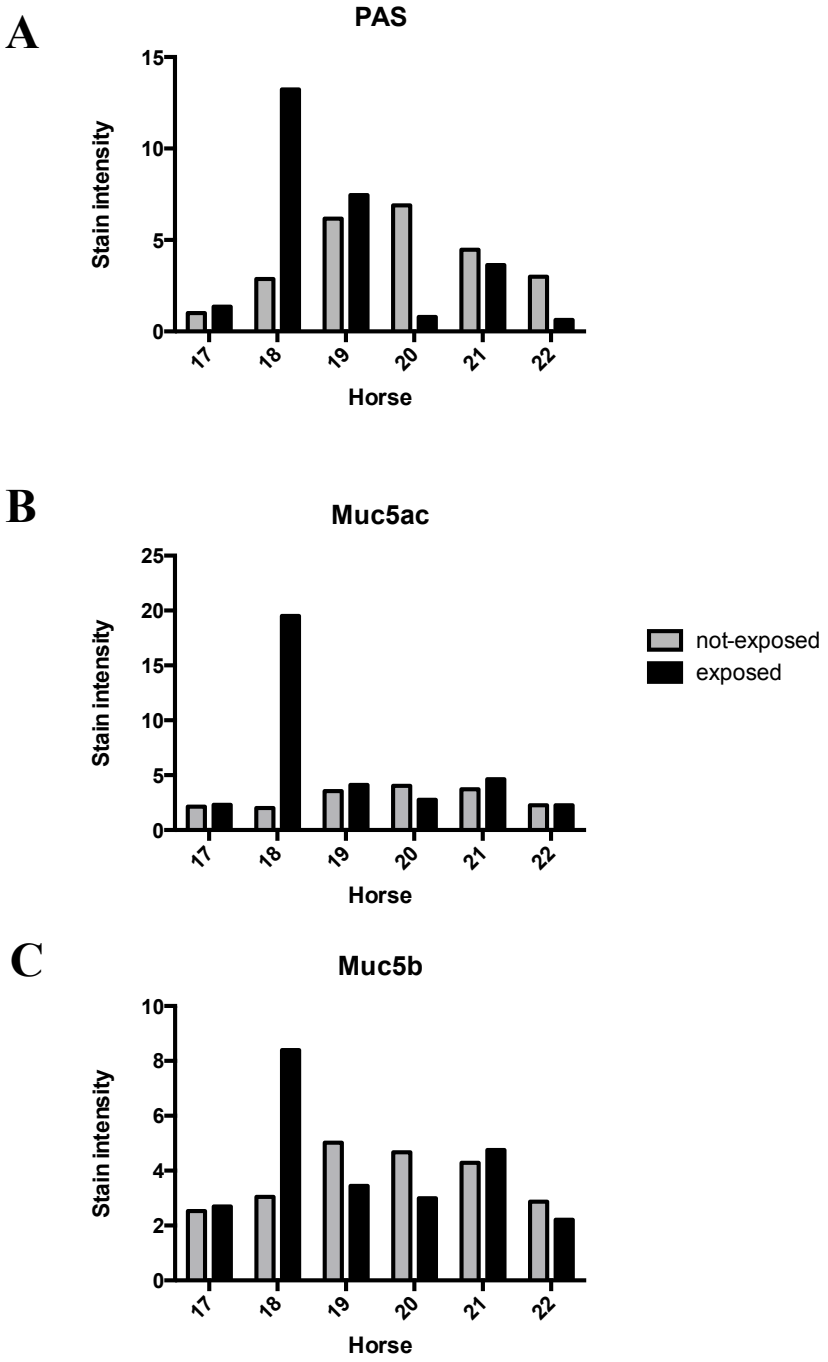


Fig. 3.9 **MUCIN IDENTIFICATION IN EQUALLY DILUTED GuHCl SOLUBILISED MUCUS SAMPLES FROM RAO-DISEASED HORSES**. **A.** Slot blot PAS stain intensity **B**. Slot blot Muc5ac immunodetection stain intensity **C**. Slot blot Muc5b immunodetection stain intensity. Numbers 17 - 22 refer to individual RAO-diseased horses sampling before (not-exposed, grey bars) and after 30 days' exposure to a dusty environment (black bars). Data not shown for control horses because their mucus did not produce a positive stain response. No significant difference (P > 0.05, Mann-Whitney U) in stain intensity between not-exposed and exposed conditions for any staining technique.



3.2.3.2 Muc5b AND Muc5ac IDENTIFICATION AND ELECTROPHORETIC MOBILITY IN MUCUS SAMPLES FROM RAO AND CONTROL EXPOSED AND NOT-EXPOSED HORSES

TW mucus was collected on our behalf by our collaborators trans-endoscopically via infusion of 100 mL sterile 0.9 % saline with suction retrieval from the control and RAO horses (collected during not-exposed and exposed conditions). The mucus samples were solubilized in 8 M GuHCl. The amount of GuHCl required to solubilise the mucus varied between samples (range 1:2 to 1:10.8 dilution); each sample had a calculated volume of 8 M GuHCl added to bring all of the samples to an equal dilution (that of the most difficult to solubilize sample) (refer to *Appendix I: Table AI.7*). Slot blotting of an aliquot from each equally diluted sample and mucin detection using PAS stain, and immunodetection for equine Muc5b and Muc5ac were performed. Mucin was not detectable in the control horse samples but was detected for the RAO horse samples, with no overall significant difference ($P > 0.05$, Mann-Whitney U) in stain intensity between asymptomatic (not-exposed and symptomatic (exposed) RAO horses (*Fig. 3.9*).

Aliquots of the equally diluted mucus samples were dialysed into 6 M urea, reduced and alkylated and used to perform agarose gel electrophoresis and western blotting onto nitrocellulose membrane followed by immunodetection for equine Muc5b and Muc5ac (*Fig. 3.10*). There was very little Muc5ac immunodetection for the samples from the control horse not-exposed samples, with only horse 16 having a faint positive band; there was a stronger Muc5ac positive band present in the exposed control samples for horses 15 and 16 only. Muc5ac detection in the RAO horse samples showed very limited faint detection in the not-exposed samples for horses 17 and 22; whilst there was a stronger positive band for Muc5ac in the RAO horses 18, 19 and 21 in their exposed environment mucus samples. There was virtually no Muc5b detection in the not-exposed mucus samples from all of the control horses (11-16) with only very faint bands being visible. The band intensity for Muc5b for the control horse exposed samples was increased slightly, notably for horse 15, although the bands remain faint. Muc5b was more readily detectable in the gel blots from the RAO horse mucus samples compared to the control horse samples, although it remained undetectable in the not-exposed mucus for horse 17, and the exposed environment mucus samples horses 17, 20 and 22. RAO horse 18 was the only horse to have a more intense Muc5b band for the exposed compared to the not-exposed samples.

A faster migrating species of Muc5b is identifiable in those horses which have more intense visible bands for Muc5b, namely horses 15 (control), 19 and 20 (RAO) in their not-exposed mucus samples, and horse 18 (RAO) in its exposed mucus sample (*Fig. 3.10*). The Agarose gel blot data for Muc5ac does not agree very well with the slot blot data (which is only available for the RAO diseased horse mucus) except for horses 17 and 22, which had very little positive stain during both exposure states. The slot blot and agarose data for the RAO horse mucus is more in agreement for Muc5b, where horses 17 and 22 had little positive staining and horse 18 has the strongest stain intensity in the exposed sample, and horses 19, 20 and 22 all had greater stain intensity in the not-exposed compared to the exposed samples on both the slot blots and the gels. This greater agreement between the 2 mucin identification methods for Muc5b than Muc5ac is likely to be a reflection of the combined effects of greater sensitivity of the eqMAN5ac antibody versus the greater abundance of Muc5b, being the predominant mucin, in the mucus samples.

3.2.3.3 SIZE DISTRIBUTION OF MUCINS FROM RAO AND HEALTHY CONTROL HORSE TRACHEAL MUCUS: SEPARATION BY RATE ZONAL CENTRIFUGATION

In order to investigate any size difference in Muc5b and Muc5ac between the 4 groups of horses, rate zonal centrifugation of GuHCl solubilised airway mucus samples from a control horse (16) and an RAO horse (19) collected under both not-exposed and exposed conditions was performed. Samples were chosen from these horses for this experiment because they had positive immunodetection for Muc5ac and Muc5b on their agarose gel electrophoresis blots. The fractions of increasing density were slot blotted and probed for mucin presence using PAS and immunodetection for equine Muc5b and Muc5b stains (*Fig. 3.11*). Mucin detection across the range of fractions for each individual horse demonstrated the typical polydisperse size nature of the equine airway mucins Muc5b and Muc5ac, with no significant difference ($P > 0.5$, Mann-Whitney U) of mucin size across the fraction range between any of the 4 groups for either mucin.

Fig. 3.10 **AGAROSE GEL ELECTROPHORESIS AND MUCIN DETECTION OF EQUALLY DILUTED GuHCl SOLUBILISED MUCUS SAMPLES AND GRAPHS DEPICTING STAIN INTENSITY.** **A**. Muc5ac **B**. Muc5b. Numbers 11 - 16 refer to individual control horses, numbers 17 - 22 refer to individual RAO-diseased horses sampled before (not-exposed) and after 30 days' exposure to a dusty environment. Grey bars on graphs refer to not-exposed mucus samples and black bars to exposed mucus samples.

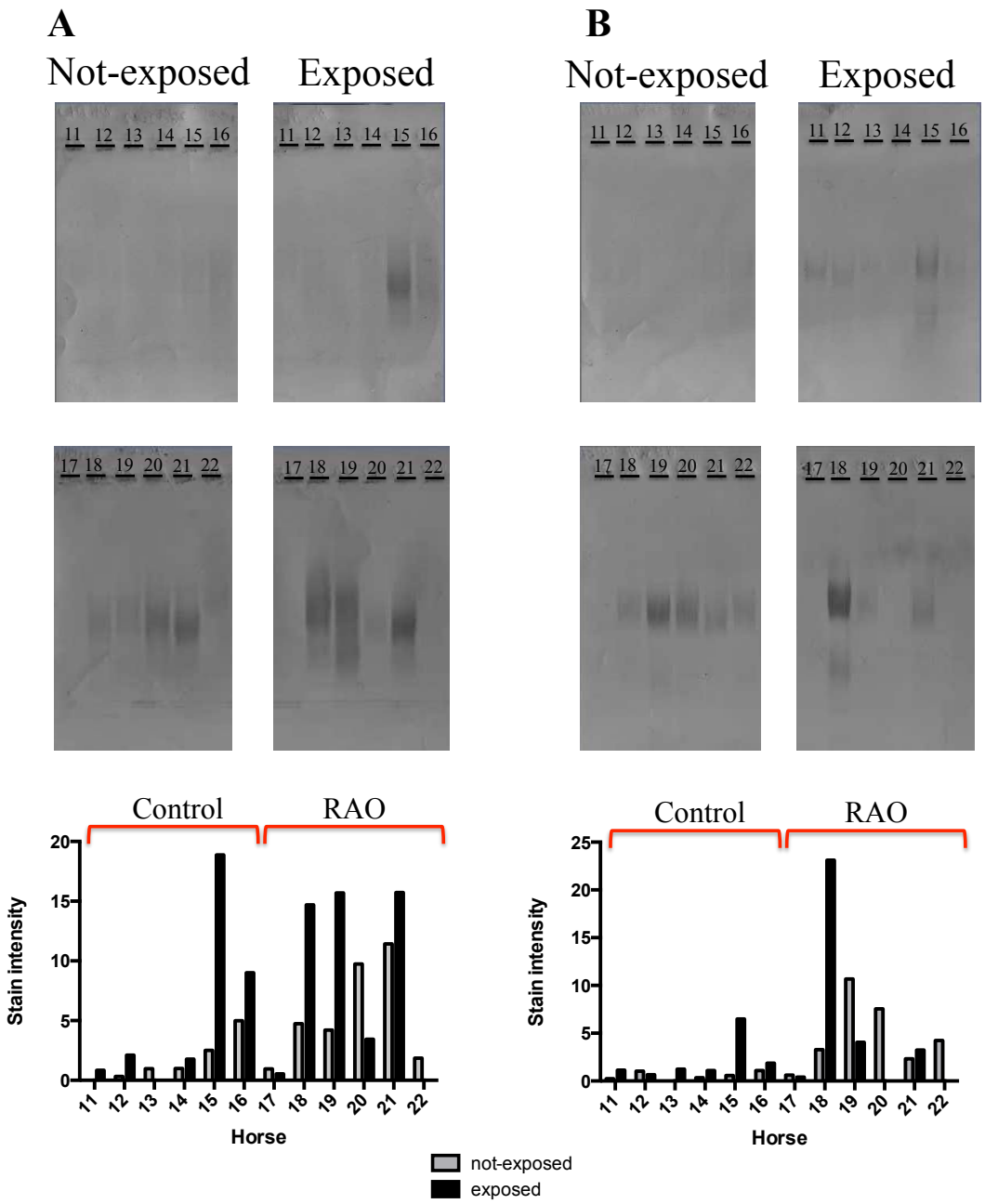
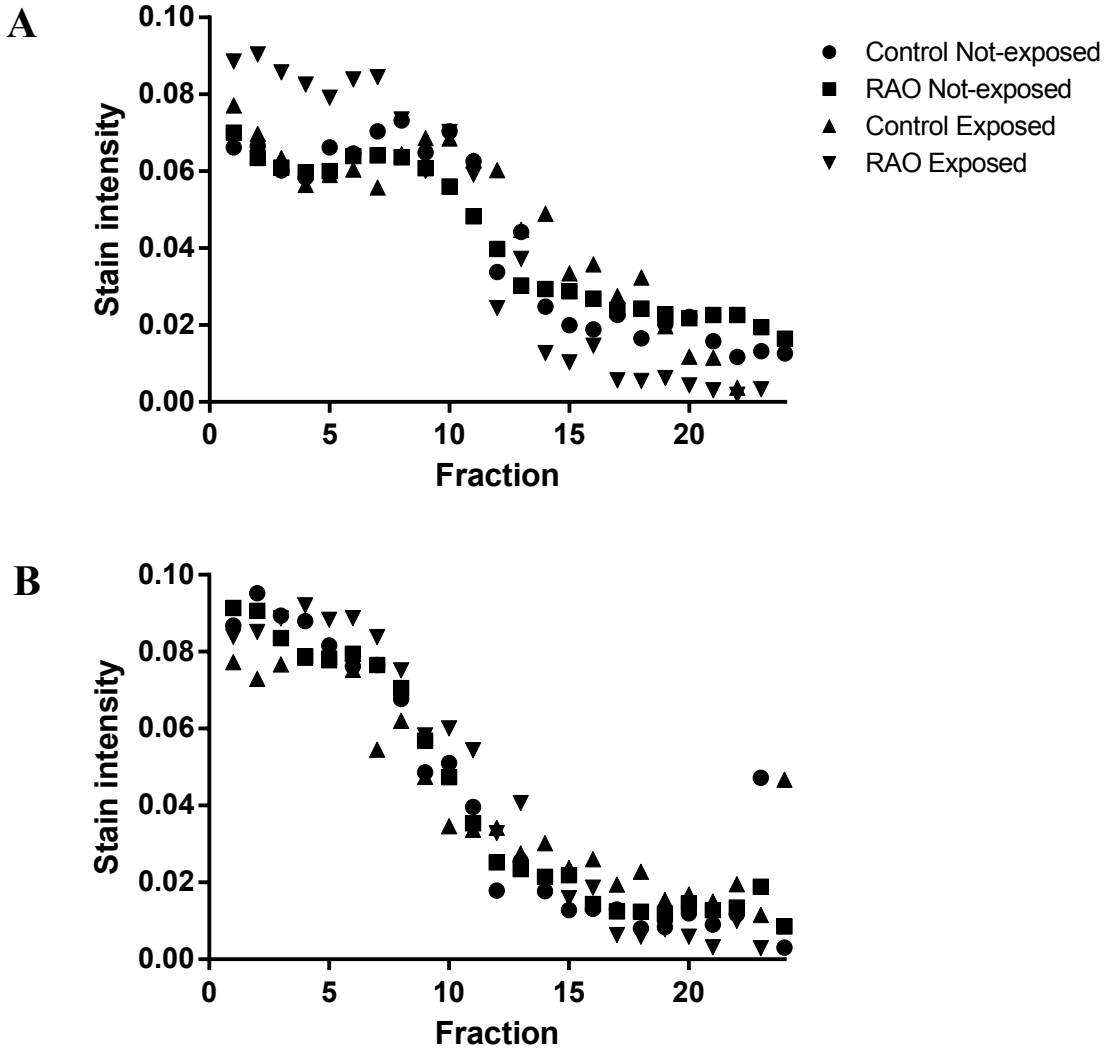


Fig. 3.11 **MUCIN SIZE DISTRIBUTION OF MUCUS FROM EXPOSED AND NOT-EXPOSED RAO AND HEALTHY CONTROL HORSES: SEPARATION BY RATE ZONAL CENTRIFUGATION.** GuHCl solubilised airway mucus samples were loaded onto a preformed GuHCl gradient and centrifuged in a Beckman SW40 swing our rotor at 4 ^{0}C 40,000 rpm for 2.5 hours. 24 fractions of increasing density were collected. Slot blot and immunodetection for equine Muc5b and Muc5ac were performed for each fraction. Graphs show mean for each group of horses (control exposed and not-exposed, RAO exposed and not-exposed). **A**. Muc5b **B**. Muc5ac. No significant difference (P > 0.5, Mann-Whitney U) for any stain types between exposed and not-exposed control or RAO samples.



3.3 DISCUSSION

The initial biochemical analysis performed on RAO airway mucus was from clinical cases of RAO, as these were the most readily available mucus samples, given that endoscopy is routinely used in the initial diagnosis of RAO. However due to the nature of clinical work, the samples were not well controlled, the animals were from a variety of management conditions and states of disease; and as sampling was performed by a number of different veterinarians, sampling technique was not necessarily identical each time. It was also impossible to have access to clinical samples from healthy horses that did not have RAO as the invasive nature of the sampling technique could not be ethically justified.

The assessment of these clinical RAO mucus samples was useful for establishing that TW mucus samples are generally more useful than BAL samples for the techniques employed here for studying mucins; and also for identifying that the airway mucins Muc5b and Muc5ac appear to be the predominant secreted equine airway mucins in RAO as is the case for healthy equine airways (Rousseau *et al.*, 2007).

Historically we know that RAO horses have an increased amount of tracheal mucus accumulation compared to healthy horses, even when they are not showing clinical signs of disease (Christley *et al.*, 2001; Robinson *et al.*, 2003). We also know that a major part of the disease pathology is that exposure to a disease-inducing environment leads to airway mucus hypersecretion (McGorum *et al.*, 1993a; Pirie *et al.*, 2001). The experiments examining mucus from exposed and not-exposed RAO diseased horses were carried out to investigate if there was a difference in secreted mucins in RAO horses when clinical signs of the disease are present or silent. Surprisingly, given that exposure leads to mucus hypersecretion, there was no statistically significant increase in mucus accumulation score between the exposed and not-exposed groups of RAO horses in this experiment. Examining the data there is a trend for a higher mucus score in the exposed horse group, but the low number of horses may be the reason that this does not reach statistical significance.

Although the identical technique was used for TW mucus sampling with identical operator and equipment in every case, the volume retrieved still varied between horse, and this will confound all results when looking for inter-horse concentration comparisons. All we can say is that when employing same technique, different amounts of mucins are retrieved. But this is a severe limitation to the study. The fact that there is little / no correlation of agarose mucin stain intensity with mucus score is a reflection of the sampling technique not retrieving a representative sample for mucin analysis. This is in

contract to previous findings by Rousseau *et al*. (2011b) where mucin content of TW samples correlated well with subjective tracheal mucus accumulation score; however the horses in that study did were healthy controls and horses with IAD, and the large majority of horses had a low amount of tracheal mucus. The study also employed a different tracheal mucus score scale of 1 - 3 and had a much larger sample size, which may go some way to explaining why they, unlike us here, were able to correlate mucin content of mucus with tracheal mucus score. Due to the issue of variability of sample dilution, it would be worth doing intra- and inter-horse comparisons of the ratio of Muc5ac to Muc5b, since this should be unaffected by variable recovery.

The immunodetection on the agarose gel blots, along with the mass spec data from the experimental RAO horses confirmed that Muc5b and Muc5ac are the main secreted polymeric mucins responsible for equine airways mucus structure in horses suffering form RAO; as had been suggested in the early experimental data on the clinical case samples. However, a large variation in mucin detection existed between the samples from different horses, despite the much more tightly controlled experimental mucus collection method (carried out in an identical way each time by the same operator). It may well be the case that there is individual variation in mucus mucin content between horses; but we are unable to conclude this from these data, as it is entirely plausible that the collection technique employed does not lead to acquisition of a representative sample of tracheal mucus. This is reflected in the fact that the mucin stain intensity measurements on the agarose gel blots do not correlate at all with the visual tracheal mucus accumulation score assigned to the horse the same time as mucus collection was performed. This is especially true with the horses that had no detectable mucins in their mucus samples but had visible mucus in their airways.

There was a faster migrating species of Muc5b identified on agarose gel electrophoresis, which is also the case for human MUC5B, where a higher charge glycoform exists (Thornton *et al.*, 1997). There was very little detectable Muc5b from healthy control horses in these experiments, but the faster migrating Muc5b band did appear on one of the control horse gels. As this was only observed in one horse, it is impossible to speculate whether this higher charge glycoform is present universally in equine airway mucus or more prevalent in the mucus of RAO diseased horses, where it has been more commonly observed in these experiments. Certainly, the faster migrating Muc5b species is seen in mucus from RAO horses both when they are not exposed and during exposure to a disease-inducing environment. The horses that had the strongest

Muc5b signal on their electrophoresis blots (experimental herd RAO horses 2 and 4) were the two horses that had the faster migrating bands, which was a reproducible finding. Whether this band only detected in these 2 horses is purely a reflection of the amount of Muc5b present in the samples or that it is not present in all horse mucus samples, we do not know.

The equine Muc5b antibody has been previously shown to be far less sensitive than the equine Muc5ac antibody (Rousseau *et al.*, 2011b), so the stain intensities of the two mucins on nitrocellulose membranes are not equally comparable. The MANeq5ac-1 antibody can detect between 10 – 1000 ng of Muc5ac, whilst the MANeq5b-1 antibody can detect between 0.1 – 10 μg of Muc5b (Rousseau *et al.*, 2011b). Thus the MANeq5ac-1 antibody is much more sensitive, so if an equal intensity of stain is noted, there will be around twenty fold times more Muc5b in the sample than Muc5ac (Rousseau *et al.*, 2011b). There was therefore far more Muc5b than Muc5ac in the mucus samples from both RAO and control horses. This coincides with the mass spec data from the same samples, identifying the presence of portions of the Muc5b and to a lesser extent Muc5ac sequences. Other mucin sequences were in the searched database, including Muc2 and Muc6, and no peptides were identified from these polymeric mucins.

There did appear to be a variability of mucin detection in MS, which may again be due to a combination of factors, chiefly the variability in mucin recovered due to sampling technique, but also due to individual variabilities in mucin amounts. The processing of MS samples may also have lead to loss of detectable mucin.

On mucin size distribution analysis of the experimental RAO horse mucus samples by rate zonal centrifugation, the mucins were polydisperse in size, as would be expected for secreted airway mucins and has been shown to be the case in samples from healthy horses (Rousseau *et al.*, 2007). There was no significant difference in Muc5b or Muc5ac size distribution across the rate zonal preformed density gradient between control and RAO horses either before or after exposure to a disease-inducing environment. Therefore the mucins do not appear to alter in their size distribution in disease and the most likely scenario is that there are simply more mucins present in RAO horse airways mucus.

3.4 CONCLUSION

From this data we can conclude that Muc5b and Muc5ac are the secreted equine airway mucins in RAO as well as in healthy horses; with Muc5b being the predominant mucin in disease as well as health. The secreted mucins from RAO patients to not appear

to be different in polydisperse size from those of healthy animals. Mucus samples collected by BAL are too dilute for biochemical anaylsis methods currently employed; and TW samples are not necessarily representative of the mucus present in the airways.

CHAPTER 4: SITES OF MUCIN PRODUCTION IN THE EQUINE AIRWAYS

4.1 INTRODUCTION

In the preceding chapter we established by examination of mucus samples from diseased horse airways that in RAO mucus, Muc5b is the predominant polymeric equine airway mucin with Muc5ac also present. Shortcomings of the preceding experiments were largely due to sample collection methods not retrieving representative mucus samples from patients, and from uncontrolled variables (such as genetic and environmental factors) between samples. One future aspiration will therefore be to produce a much more rigorously controlled reproducible mucus collection procedure in order to examine RAO mucus properties more thoroughly and compare it to healthy horse airway mucus. One way to examine genetic variability and to tightly control environmental factors would be to establish a cell culture system growing mucin-producing airway epithelial cells from donor RAO-diseased horses and studying the mucus produced. In order to be able to establish a cell culture system, we first need to know where in the horse airways is the best place to retrieve cells from. Knowledge of the anatomical location and variation of polymeric mucin producing cells and any differences that occur between diseased and healthy horses will ultimately also help our understanding of this disease in which mucus hypersecretion is a key pathological component.

What is yet to be fully established is where in the horse airways the polymeric mucins Muc5b and Muc5ac are produced, both in health and disease. It is well known that secreted mucins are produced in specialised epithelial goblet cells and in deeper sub-mucosal glands in mammalian airways (Hovenberg *et al.*, 1996a; Jeffery and Li, 1997; Rose and Voynow, 2006; Thornton *et al.*, 2008; Wickstrom *et al.*, 1998). The human airway polymeric mucins MUC5B and MUC5AC, orthologues of the equine airway mucins, are produced largely in separate cells; MUC5B primarily being made in mucous cells in the glands, whilst MUC5AC is the mucin produced in the epithelial goblet cells (Hovenberg *et al.*, 1996a; Wickstrom *et al.*, 1998). Primary data from our laboratory showed that this is not the case for equine mucins, and that both Muc5b and Muc5ac are produced in the epithelial goblet cells and the mucosal glands (Rousseau *et al.*, 2011b).

There is scant data available concerning the distribution of epithelial goblet cells in the equine airways. An anatomical investigation of the equine tracheal mucous glands of healthy horses has been performed; however mucin production in surface epithelial cells was not examined (Widdicombe and Pecson, 2002). Epithelial goblet cells have been

documented in equine airways down from trachea to bronchial level (Pirie *et al.*, 1990a; Rousseau *et al.*, 2011b), but the distribution of cells has not been not fully determined, nor the Muc5b and Muc5ac distribution identified for the healthy or RAO-diseased horse. Anatomical differences in mucin-cell distribution have been noted in the trachea, with fewer glands and epithelial goblet cells being observed in the dorsal compared to ventral trachea of normal horses (Pirie *et al.*, 1990a; Widdicombe and Pecson, 2002). However these studies either did not examine the entire length of the trachea, had a small sample size, or only looked at one type of mucin-producing cell.

Preliminary data from our laboratory have identified a large variation of epithelial mucin-cell number per length of epithelium amongst healthy horses, which needs to be further investigated and quantified before comparisons can be made with diseased horse airways (Rousseau *et al.*, 2011b). As previous data has shown that there is a slightly lower number of mucin cells in the dorsal trachea, (Pirie *et al.*, 1990a; Widdicombe and Pecson, 2002), it is important to establish any differences in goblet cell distribution along the length of the trachea, with respect to future sampling sites for cell culture experiments, in order to be able to target an area where the highest yield of goblet cells can be achieved, or at least to know that cells collected would be of a standard phenotype if sampled from a particular area. It could also help us to target therapeutics in the future if there is one part of the respiratory tract where mucin-producing cells predominate. The equine trachea is quite sizable and therefore it is important to know if sampling along any point makes a difference to the goblet cell yield. It is impossible to know if mucin production sites alter in disease until the normal mucin production sites, size and distribution variables from a population of healthy horses have been quantified. Therefore in this chapter we carry out a histological investigation of healthy horse airways in order to establish healthy horse mucin-cell size, number and distribution, and examine variation between individual healthy horses.

We examined the mucin-cell size, number and distribution (epithelial goblet cells and sub-mucosal glands) at various points along the length of the trachea and 3 generations of bronchi in airways from 9 healthy horses (AH1 – AH9), fit for human consumption and showing no signs of respiratory disease. In order to investigate if epithelial goblet cells are evenly distributed throughout the respiratory tract, we examined the number of goblet cells present per 1000 μm length of epithelium and also their mean size, as well as the sub-mucosal gland size and percentage containing mucin-stained positive material. We performed this on identical sections from each horse's airway, including: the cranial,

middle and caudal portion of the trachea, considering the circumferential ventral, dorsal, left and right sections separately; comparison of trachea with the bronchial sections and comparison between the primary, secondary and tertiary bronchi (refer to *Methods*). We used a general mucin stain and then for serial sections of tissue, developed a technique using mucin-specific antibodies to examine the distribution of Muc5b and Muc5ac across the equine airways.

As stated previously, horses with RAO have increased secreted airways mucus compared to normal horses both during challenge when acute stages of the disease are apparent, and in clinical remission (Robinson *et al.*, 2003). The polymeric secreted mucins are the structural components of mucus; ergo it is expected that mucin hypersecretion occurs in RAO. It is not fully understood if the mucin cells are increased in size or number or secretory activity in RAO, and a histological study of their number and distribution in RAO horses with a comparison to the situation in healthy horses will help us to understand this further.

In order to do this we performed histological examination of airways sections from RAO and control horses either exposed to challenge environment or not at time of euthanasia. We were kindly provided with samples from a tissue bank at The University of Montreal in order to be able to complete this part of the project. The stored tissue samples had all been collected from identical areas of the lung (L samples, containing bronchi) and the distal trachea (T) (refer to *Methods*). We examined identical parameters as for the healthy horse histological study, i.e. the number of goblet cells present per 1000 μm length of epithelium and also their mean size, as well as the sub-mucosal gland size and percentage containing mucin-stained positive material. We compared the anatomical locations, lung versus trachea, for both healthy and RAO-diseased horses and also for exposed and not-exposed environmental conditions for both anatomical locales.

The periodic-acid Schiff's and alcian blue (PAS AB) stain is useful for identification of mucin-containing cells, being a general stain, but does not differentiate between individual types of mucin (Muc5b and Muc5ac in the case of equine airway secreted polymeric mucins). Identification of the two major equine airway mucins separately in equine airway tissue samples requires immunohistological techniques using antibodies directed at each specific mucin. Before mucin-specific staining of tissue sections could be performed, we first needed to develop tissue specific Muc5b and Muc5ac

antisera, which is covered in the first part of the chapter, after which the results of the healthy horse and then the comparative RAO versus healthy control horse histological studies are discussed.

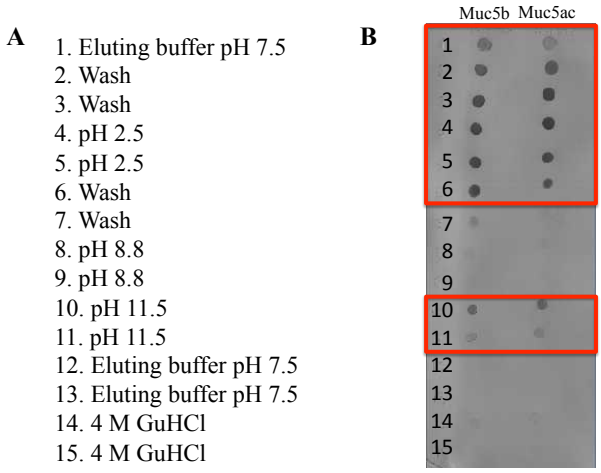
4.2 RESULTS

4.2.1 ANTIBODY PREPARATION

4.2.1.1 AFFINITY PURIFICATION OF Muc5b and Muc5ac SPECIFIC ANTIBODIES

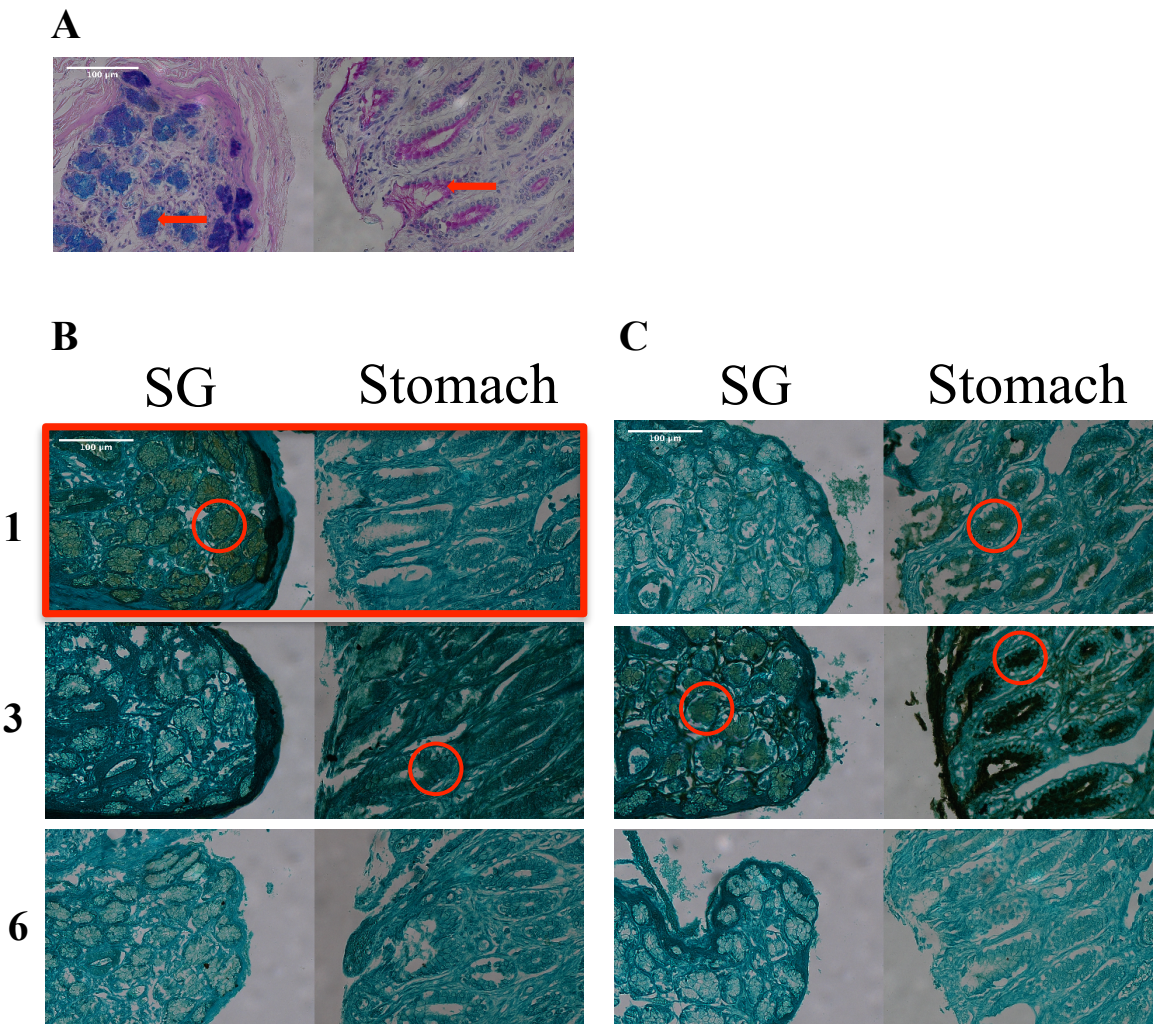
In order to perform immunohistochemistry detecting equine Muc5b and Muc5ac in equine airway tissues, affinity purification of the polyclonal antisera (eqMAN5ac-I, eqMAN5b-I; refer to *Chapter 2: Methods*) was necessary to ensure antibody tissue specificity on histological sections. This is because even though these antisera are sufficiently specific for immuno-identification of the Muc5ac and Muc5b mucins in biochemical samples, they may give false positive staining on histological sections. Using affinity purification on Protein-A solubilised on Sepharose CL-4B beads, we produced a series of fractions by varying the pH of the elution buffer (refer to *Methods*). Each of the subsequent 15 fractions for both eqMAN5ac-I and eqMAN5b-I were then tested for antibody content on dot blot (5 µL/ dot) and probing with alkaline phosphatase labelled secondary antibody and BCIP/NBT colour detection. This showed that fractions 1 - 6, 10 and 11 for both antisera had a positive response (*Fig 4.1*).

Fig. 4.1 **ANTIBODY AFFINITY PURIFICATION**. Affinity purification of the polyclonal antisera (eqMAN5ac-I, eqMAN5b-I) created 15 fractions of varying pH as outlined in **A.** **B.** Dot blot of fractions (5 µL per dot) onto nitrocellulose membrane and probing with a peroxidase-labelled secondary antibody and colour detection using BCIP/NBT revealed reactive fractions (outlined by red boxes) for testing tissue specificity.



The positive fractions were then used to test specificity using positive and negative control tissue for each antibody. Equine parotid salivary gland was the positive control for Muc5b and negative control for Muc5ac, whilst equine stomach was the positive control for Muc5ac and negative control for Muc5b, as used previously (Rousseau *et al.*, 2011b) (*Fig.4.2; AppendixII: Fig. AII.2*). A PAS AB stain (with haematoxylin counterstain) of a section from both tissues was prepared for the purpose of identification of mucin-positive tissue, which is mixed acidic and neutral (blue/purple) in the salivary gland and neutral (red/pink) in the stomach (*Figs. 4.2, 4.3, 4.4*). Each antibody fraction was used at a concentration of 1:100 in block solution, incubated overnight, and detected using DAB colour detection (brown colour) and counterstained using fast green stain (1 % (w/v) fast green in acetic acid, for 60 seconds) (*Fig. 4.2; AppendixII: Fig. AII.2*). For both antisera, fraction 3 was unsuitable due to positive staining in the negative control tissue; whilst fractions 6, 10 and 11 were ruled out due to lack of any positive mucin staining in positive control tissues for both antisera (*Fig.4.2; AppendixII: Fig. AII.2*). The remaining fractions, 1, 2, 4 and 5 from the eqMAN5b-I antiserum were positive in the salivary gland and negative in the stomach tissue mucin-cell areas and therefore considered possible candidates for containing specific antibodies for eqMuc5b for use in subsequent studies on equine respiratory epithelial tissue sections. For eqMuc5ac, positive staining of positive control tissue (stomach) was detected in fractions 1, 2, 4 and faintly in 5; and although none of those fractions had positive stain uptake in the mucin-cell areas of the negative control tissue (salivary gland), there was some positive background staining of the tissues (*Fig. 4.2; AppendixII: Fig. AII.2*). Thus the eqMuc5ac fractions required further investigation to identify a possible suitable tissue-specific fraction. The fast green counterstain used made it quite hard to discern faintly positive areas of tissue staining on the sections, and so the following investigations were carried out in order to elicit a better method of counterstaining that would allow accurate detection of DAB stain positive cells.

Fig. 4.2 **MUC5B AND MUC5AC ANTIBODY PURIFICATION FRACTIONS: POSITIVE AND NEGATIVE CONTROL TISSUE TESTING**. Representative photomicrographs of formalin fixed equine salivary gland (SG) and stomach tissues with examples of affinity purified fractions of equine mucin polyclonal antisera (eqMAN5ac-I, eqMAN5b-I). **A**. PAS AB general mucin stain (haematoxylin counterstain) of the same equine tissues for identification of mucin-positive areas of tissue (red arrows). **B**. Muc5b antibody fractions **C**. Muc5ac antibody fractions. Slides made from serial sections of paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection and fast green (1 % w/v) counterstain. Numbers refer to affinity purification fractions as described in *Fig 4.1*. Red circles indicate examples of positive staining mucin tissue. Red box highlights an example of a Muc5b labelled section used in future investigations. Scale bar 100 µm; original images captured at x 40 magnification.



4.2.1.2 COUNTERSTAIN FOR IMMUNOHISTOCHEMISTRY

The fast green counterstain had initially been used based on techniques previously employed in our laboratory for mucin immunohistochemistry (Hasnain et al, 2010). However, in these sections the vivid nature of the fast green stain used was so bright that it made detection and thus analysis of the DAB positive material difficult (*Fig. 4.2; Appendix II: Fig. AII.2*). In order to optimise the conditions employed for immunohistochemistry of the respiratory tissues, staining of positive and negative control tissues was repeated for purified fractions 1, 2, 4 and 5 for both antisera using the fast green counterstain at a more dilute concentration (0.5 % (w/v) for 30 seconds) and also using haematoxylin counterstain as an alternative counterstain method (1:5 dilution for 30 seconds) (*Figs. 4.3, 4.4*; *Appendix II: Figs. AII.2, AII.3*).

The more dilute concentration of fast green counterstain still yielded images in which it was difficult to discern positively staining tissue, particularly when positive staining was faint (refer to *Appendix II: Figs. AII.2, AII.3*). The haematoxylin counterstain proved superior for visualisation of DAB positive staining tissue with eqMAN5ac-I and eqMAN5b-I purified antibody fractions compared to either the original or modified fast green counterstain conditions, and haematoxylin was used as the counterstain in all future experiments (*Figs. 4.2 - 4.4*; *Appendix II: Figs. AII.2, AII.3*).

For the positive and negative control tissue stained with purified eqMuc5b antibody fractions 1, 2, 4 and 5, the expected outcome is positive DAB staining salivary gland and negative DAB staining in equine stomach. The superior visualisation of DAB positive staining permitted by the haematoxylin counterstain method revealed that whilst this was the case for fractions 2 and 5; fractions 1 and 4 also had DAB positive staining in mucin-cells of the stomach tissue and so were unsuitable for use as Muc5b antibodies (*Fig. 4.3*). Fractions Muc5b-2 and Muc5b-5 were then tested for their suitability on sections of respiratory tissue (refer to *section 4.2.1.3*).

Fig. 4.3 **EQUINE MUC5B ANTIBODY AFFINITY PURIFICATION ELUTION COUNTERSTAIN METHOD.** Slides made from serial sections of paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection. Salivary gland positive control, stomach negative control for Muc5b. **A.** PAS AB general mucin stain (haematoxylin counterstain) of the same equine tissues for identification of mucin-positive areas of tissue (red arrows). **B.** Salivary gland is positive control and stomach tissue is negative control for Muc5b. Red circles indicate examples of positive staining mucin tissue. Representative images shown for Muc5b fractions 1, 2, 4, and 5 with haematoxylin counterstain. Scale bar 100 µm; original images captured at x 40 magnification.

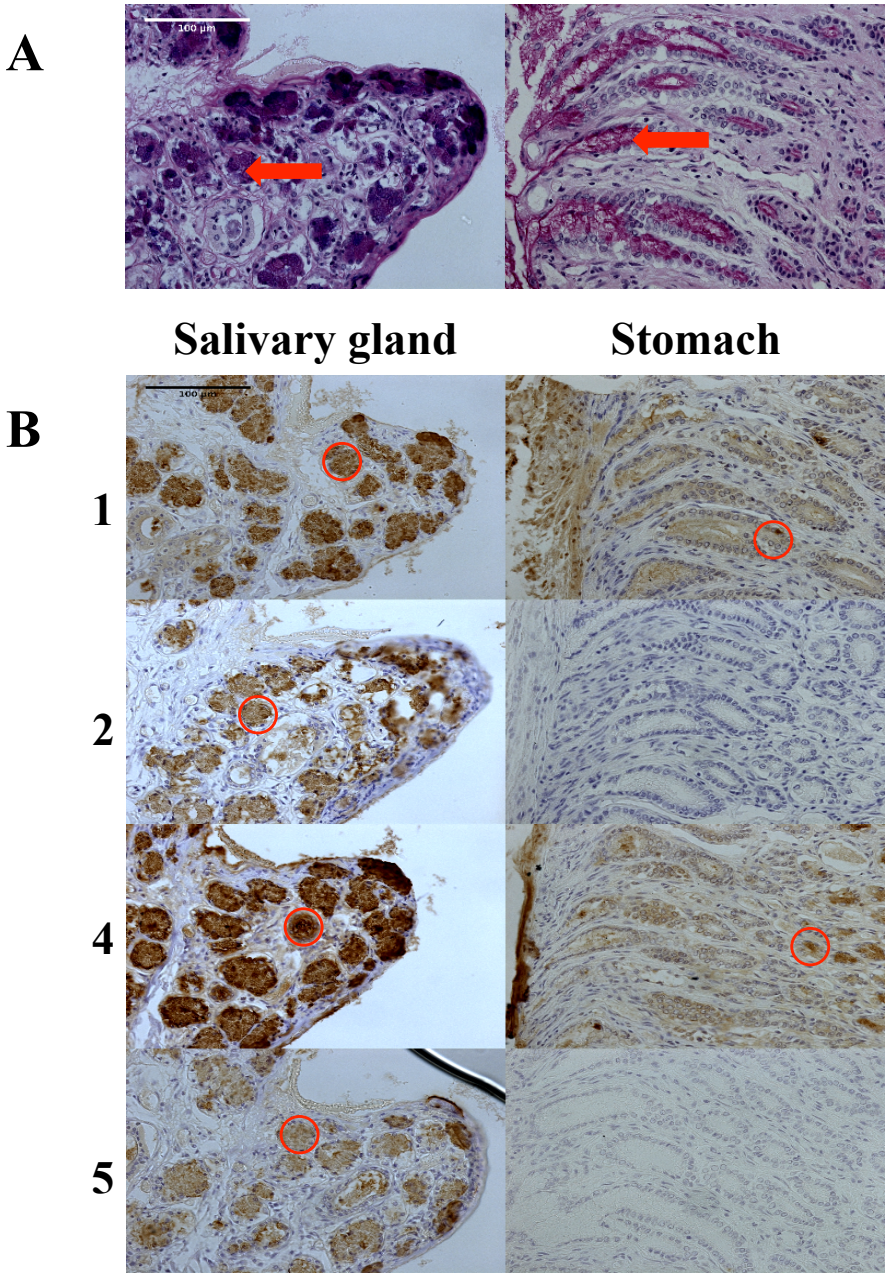
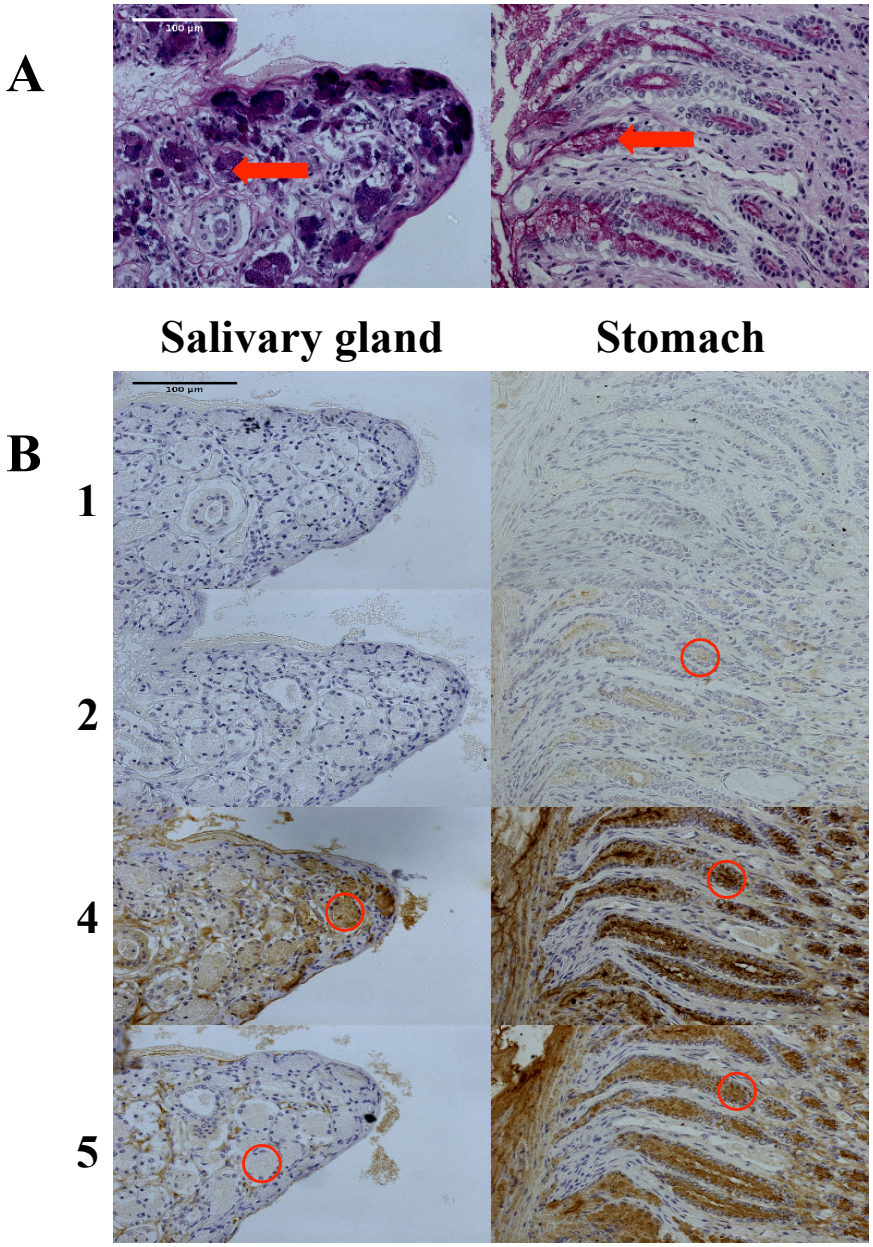


Fig. 4.4 **EQUINE MUC5AC ANTIBODY AFFINITY PURIFICATION ELUTION COUNTERSTAIN METHOD.** Slides made from serial sections of paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection. Salivary gland negative control, stomach positive control for Muc5ac. **A.** PAS AB general mucin stain (haematoxylin counterstain) of the same equine tissues for identification of mucin-positive areas of tissue (red arrows). **B.** Salivary gland is negative control and stomach tissue is positive control for Muc5ac. Red circles indicate examples of positive staining mucin tissue. Representative images shown for Muc5ac fractions 1, 2, 4, and 5 with haematoxylin counterstain. Scale bar 100 µm; original images captured at x 40 magnification.



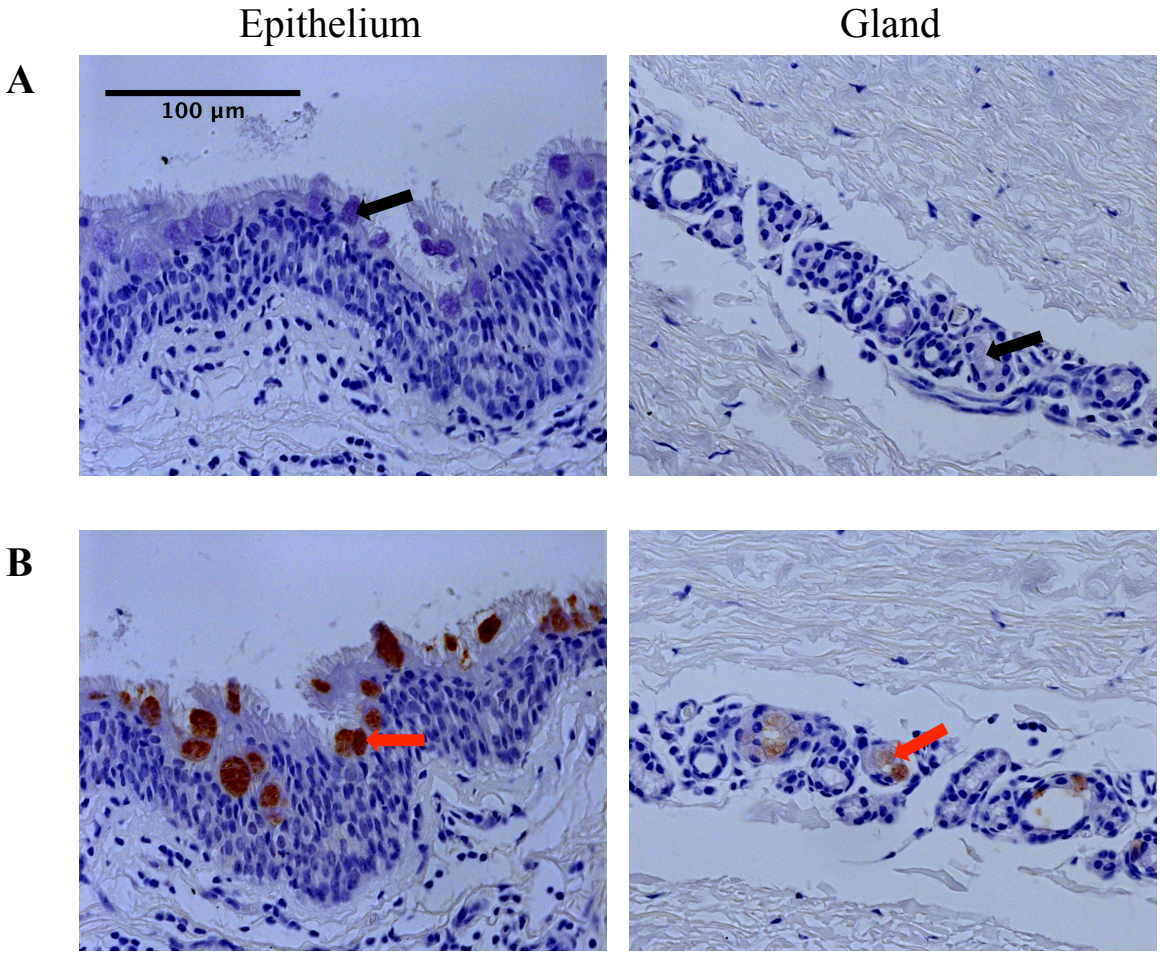
For the positive and negative control tissue stained with purified eqMuc5ac antibody fractions 1, 2, 4 and 5; the expected outcome is positive DAB stain in stomach and negative DAB staining in equine salivary gland. Results from antibody fractions from the eqMAN5ac-I antisera (1, 2, 4 and 5) counterstained with haematoxylin provided no suitable fraction for use in immunohistochemistry to specifically identify muc5ac-containing cells. There was no positive staining of stomach tissue for fraction 1, whilst fraction 4 had strong positive staining of the negative control salivary gland mucin-cell tissue, ruling both of these fractions out as suitable Muc5ac antibodies (*Fig. 4.4*). Fraction Muc5ac-5 showed a strong positive DAB staining of stomach tissue, but unfortunately also showed positive staining of background tissue in the negative control (*Fig. 4.4*). Muc5ac-2 was the only potentially useful fraction, showing a faint positive DAB staining of positive control stomach tissue and no unwanted positive DAB staining of negative control salivary gland tissue (*Fig. 4.4*). If Muc5ac-2 is to be useful for positive identification of Muc5ac in equine respiratory tissue, further investigation of the staining protocol is required.

Before getting involved in a potentially complex tangent of how to get a Muc5ac antibody working, we decided to concentrate first on the refinement of Muc5b staining technique, for which we had already identified suitable Muc5b-specific antibodies (fractions Muc5b-2 and Muc5b-5) above. The first thing to do to decide if one or both of these purified antibodies would be useful was to test them on equine respiratory tissue.

4.2.1.3 TESTING Muc5b-2 and Muc5b-5 ANTIBODY FRACTIONS ON RESPIRATORY TISSUE

Equine Muc5b purified antibody fractions Muc5b-2 and Muc5b-5 were tested at 1:100 dilution on equine respiratory tissue, with haematoxylin counterstain. The result of this was positive DAB staining of equine respiratory epithelial goblet cells and glands with the Muc5b-5 fraction but not the Muc5b-2 fraction (*Fig. 4.5*). Thus Muc5b-5 was used for all future experiments of equine respiratory tissue samples for evaluation of Muc5b producing cells in the equine respiratory tract.

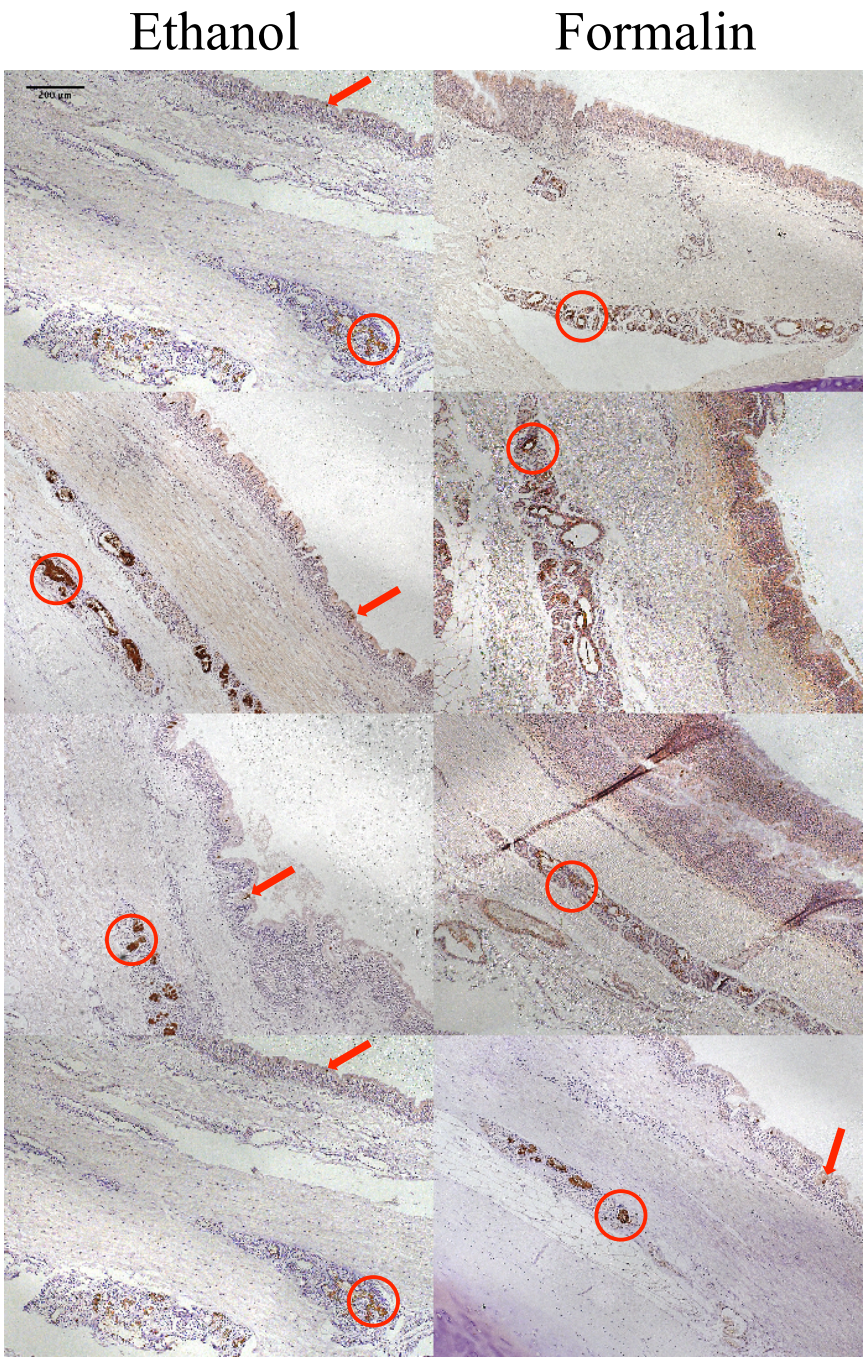
Fig. 4.5 **STAINING WITH PURIFIED ANTIBODY FRACTIONS MUC5B-2 AND MUC5B-5**. Slides made from paraffin-embedded healthy equine respiratory epithelium were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection. Counterstaining with haematoxylin. **A**. Affinity purified antibody fraction Muc5b-2. **B**. Affinity purified antibody fraction Muc5b-5. Original magnification x 40. Scale bar 100 µm. Black arrows indicate examples of negatively stained mucin-producing cells. Red arrows indicate examples of positively stained mucin producing cells.



4.2.1.4 REFINEMENT OF PROTOCOL FOR Muc5b-5 IMMUNOHISTOCHEMISTRY

Purified Muc5b-5 antibody was used (at a concentration of 1:100) with haematoxylin counterstaining in immunohistochemistry staining of a selection of both ethanol and formalin-fixed tracheal samples from one randomly selected healthy abattoir horse (AH9), in order to test the immunohistochemical staining on a number of samples before staining all sections from all horses (*Fig. 4.6*).

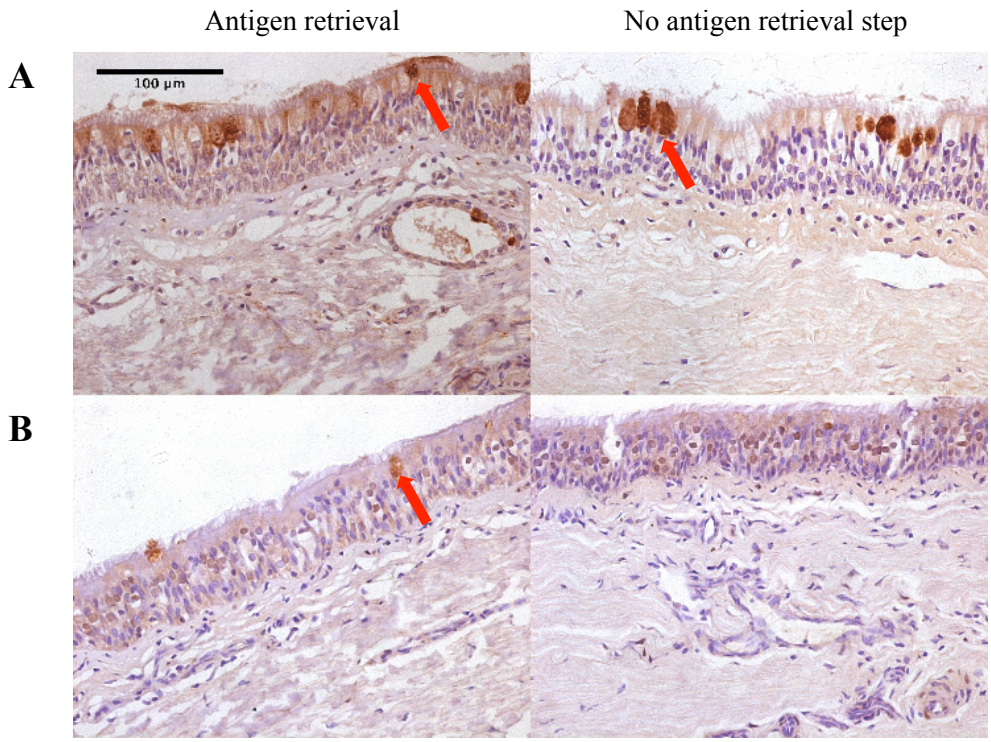
Fig. 4.6 **EQUINE MUC5B STAINING OF RESPIRATORY TISSUE: COMPARISON OF ETHANOL VS FORMALIN TISSUE FIXATION.** Slides made from paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection. Haematoxylin counterstain. Tracheal sections from one healthy horse are used. Images show representative paired ethanol and formalin fixed sections, each pair from an identical anatomical location of the trachea. Red arrows indicate positive DAB stained epithelial goblet cells. Red circles indicate DAB positive stained sub-mucosal gland tissue. Scale bar 200 µm; original images captured at x10 magnification.



Examination of images obtained from these experiments revealed a variable background of DAB staining over the entire tissue, more obvious in some slides than others from the respiratory tract. We also noted that although generally in all slides the sub-mucosal gland staining was Muc5b positive, on almost half the slides there were no identifiable Muc5b positive goblet cells, whilst these were numerous on other slides (*Fig. 4.6*). We examined paired slides (one ethanol fixed, the other formalin fixed) from identical anatomical locations. This highlighted that the majority of slides that lacked identifiable Muc5b positive goblet cells were of sections cut from formalin fixed tissue, whilst DAB positive goblet cells were identifiable in the epithelium from paired ethanol-fixed tissues (*Fig. 4.6*). The background DAB staining was present variably with both the ethanol and formalin fixed samples (*Fig.4.6*). This indicated that the ethanol fixed-tissues were superior for use for more accurate identification of Muc5b positive epithelial goblet cells.

Based on the above findings, ethanol-fixed tissue provides better goblet cell identification, but the problem of variable interference of DAB-positive background staining remains. Review of the immunohistochemistry staining protocol (refer to *Methods*) highlighted that the antigen retrieval step (involving microwave heating of slides in sodium citrate), was designed primarily for antigen retrieval of formalin-fixed tissues. We wondered if the antigen-retrieval step was producing the excessive background on the images of ethanol-fixed tissue and if removal of this step would resolve the issue. Therefore, the protocol was repeated, with and without the antigen retrieval step for both formalin and ethanol-fixed mid tracheal respiratory epithelium tissue samples from the same location in one randomly selected healthy abattoir horse (AH3) (*Fig. 4.7*). For the ethanol-fixed tissue, DAB positive mucin-producing cells were identified, but there was also excessive background positive DAB stain when the antigen retrieval step was employed (*Fig. 4.7*). For the formalin-fixed tissue, it was difficult to identify DAB positive mucin-producing cells when the antigen retrieval step was not employed, but on this occasion we could identify Muc5b positive goblet cells in the respiratory epithelium of the formalin fixed tissue with use of the antigen retrieval step (*Fig. 4.7*). We were hopeful to still be able to use the formalin fixed tissues for the project and this information indicated to us that the optimum conditions for formalin-fixed equine tissue required the antigen-retrieval citrate microwave step; whilst the ethanol-fixed tissues were better without the antigen retrieval step for Muc5b immunohistochemical staining.

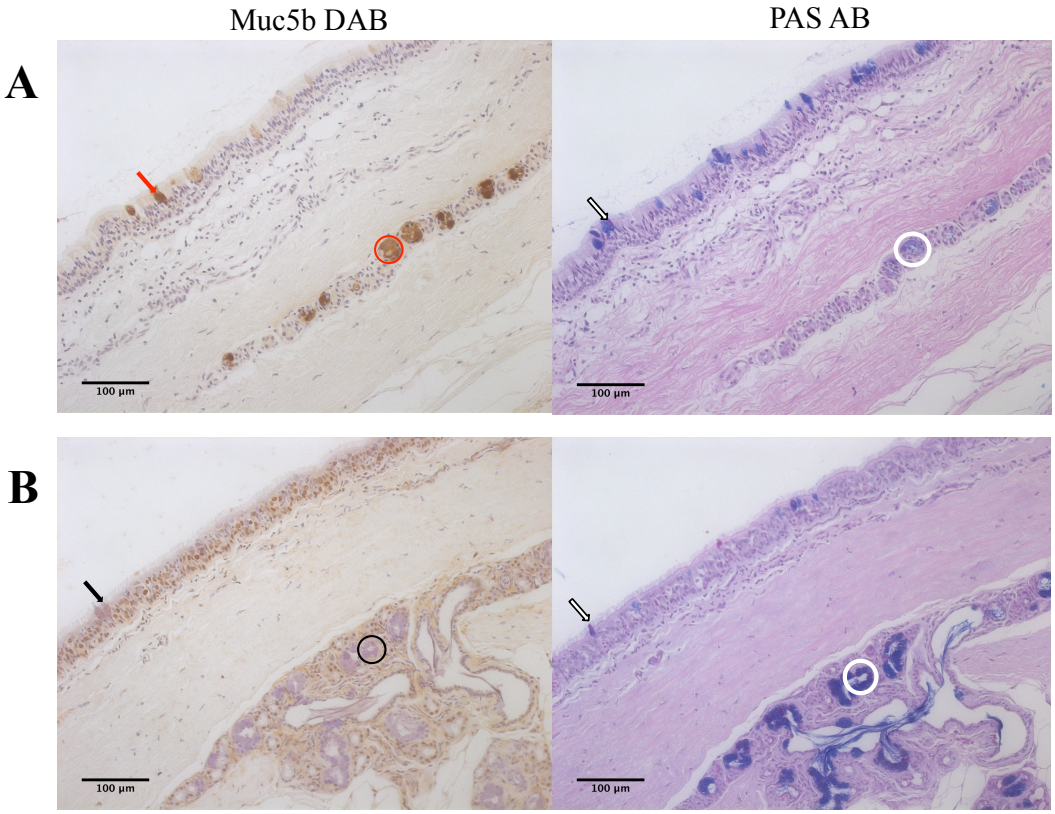
Fig. 4.7 **TISSUE FIXATION AND ANTIBODY RETRIEVAL STEPS FOR MUC5B IMMUNOHISTOCHEMISTRY DETECTION IN EQUINE RESPIRATORY TISSUE**. Slides made from ethanol-fixed and formalin fixed paraffin-embedded equine respiratory mid-tracheal epithelium from one horse (AH3), were stained using Muc5b purified antibody at 1:100 dilution incubated overnight and detected using DAB colour detection with and without antigen retrieval step (microwaved in sodium citrate). Haematoxylin counterstain. A. Ethanol fixed tissue. B. Formalin fixed tissue. Red arrows indicate positive DAB stained epithelial goblet cells. Representative images shown. Original magnification x 40. Scale bar 100 µm.



To test the above finding that ethanol fixed tissue did not require an antigen retrieval step whilst formalin fixed tissue did, we performed Muc5b immunohistochemistry on slides prepared from all 32 sections (ethanol and formalin fixed paired samples, refer to *Methods*) of the respiratory tract from the same randomly selected healthy abattoir horse (AH3). The slides were stained using purified equine Muc5b-5 antibody (1:100 dilution) with haematoxylin counterstain, following the immunohistochemistry protocol described in the methods, omitting the antigen retrieval step for the ethanol fixed tissues (*Fig. 4.8*). Slides were also prepared from the same blocks (in serial section to those prepared for Muc5b staining) and stained with general mucin stain (PAS AB, haematoxylin

counterstain) for comparison of the mucin-producing cells in the DAB stained slides (*Fig. 4.8*).

Fig. 4.8 **ETHANOL VS FORMALIN FIXED TISSUES: MUC5B AND PAS/AB STAIN MATCHED TISSUES DEMONSTRATING EPITHELIAL GOBLET CELL AND MUCOSAL GLANDS IN EQUINE RESPIRATORY TISSUE**. Slides made from ethanol-fixed and formalin fixed paraffin-embedded equine respiratory mid-tracheal epithelium from the one horse (AH3), were stained using Muc5b purified antibody at 1:100 dilution incubated overnight and detected using DAB colour detection (left-hand side images). Serial section slides were stained with PAS AB stain (right hand-side images) for comparison of glycoprotein positive staining in sections. Counterstaining with haematoxylin. **A**. Ethanol fixed tissues (no antigen retrieval step). **B** Formalin-fixed tissue (antigen retrieval step). Red arrows indicate positive DAB stained epithelial goblet cells. Red circles indicate DAB positive stained sub-mucosal gland tissue. Black arrows indicate positive DAB negative epithelial goblet cells. Black circles indicate DAB negative sub-mucosal gland tissue. Hollow black arrows indicate PAS AB positive stained epithelial goblet cells. White circles indicate PAS AB positive stained sub-mucosal gland tissue. Representative images shown (32 slides stained with each stain) Original magnification x 20. Scale bar 100 µm.



Analysis of the slides demonstrated that those from ethanol-fixed tissue with the improved protocol produced reliable DAB-positive staining of epithelial and sub-mucosal mucin-cells, with reduced DAB-positive background. Unfortunately the slides prepared from formalin-fixed tissues did not reliably have positive DAB staining of the mucin cells (*Fig. 4.8*). As the formalin-fixed tissues gave unsatisfactory results for identification of Muc5b in equine respiratory tissue, we discontinued use of formalin-fixed tissue for the investigation of mucin-producing cells in healthy horse respiratory tracts.

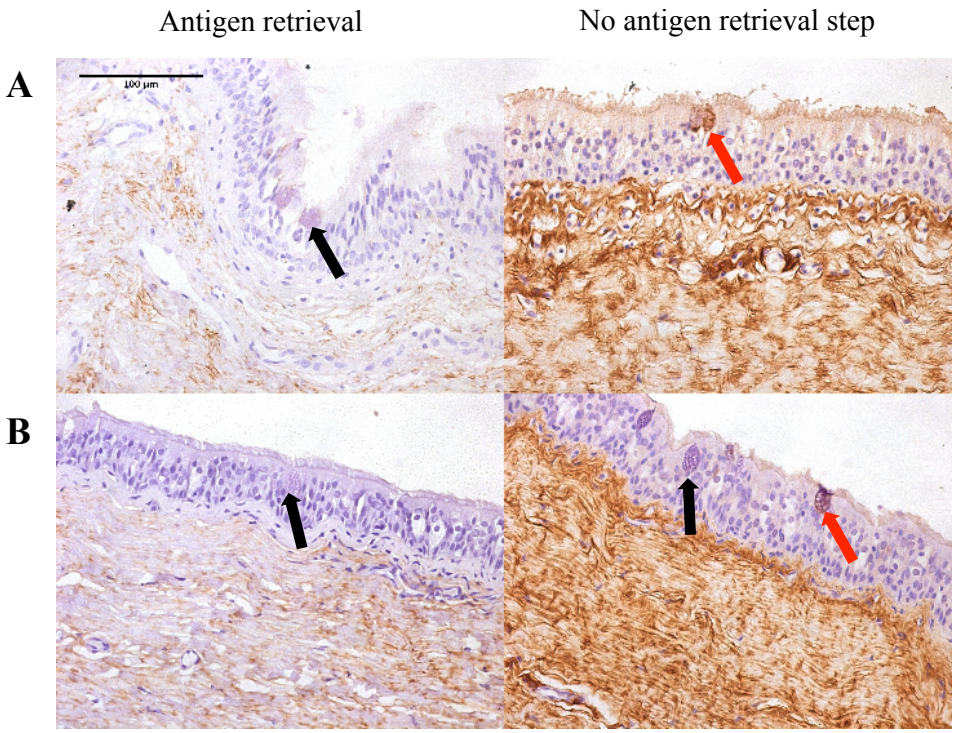
The above investigations revealed that the optimal conditions for detection of equine Muc5b on equine respiratory tissue is the use of purified antibody Muc5b fraction 5 at a concentration of 1:100 on ethanol-fixed respiratory tissues with omission of the antigen retrieval step from the immunohistochemistry protocol and with haematoxylin counterstain.

4.2.1.5 REFINEMENT OF PROTOCOL FOR Muc5ac-2 IMMUNOHISTOCHEMISTRY

We previously identified Muc5ac-2 as the purified fraction that may, with some refinement of technique, be useful for immunohistochemical identification of Muc5ac in equine respiratory tissue (refer to *4.2.1.2*). However, the positive DAB staining of positive control tissue was only faint in the images obtained so far, and so further work needed to be done to try to improve this. We had found that for the Muc5b-5 antibody that the type of tissue fixative (ethanol or formalin) and the inclusion or omission of the antigen retrieval step had an effect on the outcome of the DAB positive staining of mucin in tissues, so we decided first of all to investigate these two factors with respect to the Muc5ac-2 fraction. Purified Muc5ac-2 antibody was used (at a concentration of 1:100) with haematoxylin counterstaining in immunohistochemistry DAB staining of a selection of both ethanol and formalin-fixed tracheal samples with and without the antigen retrieval step (in serial-cut sections) from one randomly selected healthy abattoir horse (AH5) (*Fig. 4.9*). Mucin-positive DAB staining was not identified in epithelial goblet cells of the slides that underwent the antigen-retrieval step for both ethanol and formalin-fixed tissue, and so this method of tissue processing was discontinued for the remainder of our investigation of Muc5ac-2 staining (*Fig. 4.9*). However, without the antigen retrieval step,

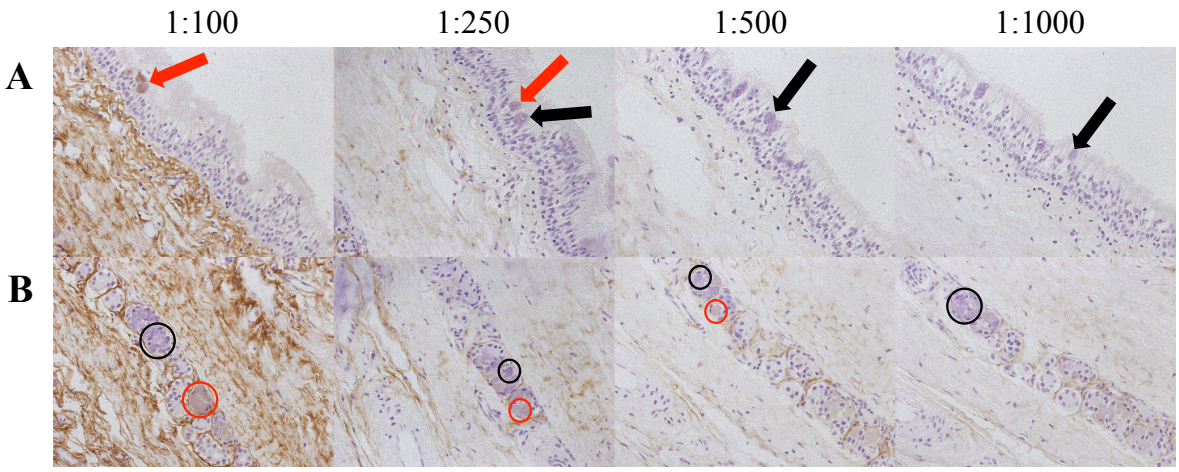
there was also a strong background DAB positive staining with both fixation methods (*Fig. 4.9*). We observed that the goblet cell staining was more consistently positive and stronger in the ethanol than formalin fixed tissues, with some notable negative DAB staining of goblet cells in the formalin-fixed tissues (*Fig. 4.9*). Therefore, ethanol fixation of tissue, along with omission of the antigen retrieval step, appeared to be the best way to proceed for our optimisation of Muc5ac-2 staining technique. However the issue of a strong background DAB positive staining needed to be reduced and so we next investigated ways to reduce the background staining whilst retaining the Muc5ac-positive tissue staining.

Fig. 4.9 **TISSUE FIXATION (ETHANOL VS FORMALIN) AND ANTIBODY RETRIEVAL STEPS FOR MUC5AC-2 IMMUNOHISTOCHEMISTRY DETECTION IN EQUINE RESPIRATORY TISSUE**. Slides made from ethanol-fixed and formalin fixed paraffin-embedded equine respiratory mid-tracheal epithelium from one randomly selected healthy abattoir horse (AH5), were stained using Muc5ac-2 purified antibody at 1:100 dilution incubated overnight and detected using DAB colour detection with and without antigen retrieval step (microwaved in sodium citrate). Haematoxylin counterstain. **A**. Ethanol fixed tissue. **B**. Formalin fixed tissue. Red arrows indicate positive DAB stained epithelial goblet cells. Black arrows indicate negative DAB stained epithelial goblet cells. Original magnification x 40. Scale bar 100 µm. Representative images shown.



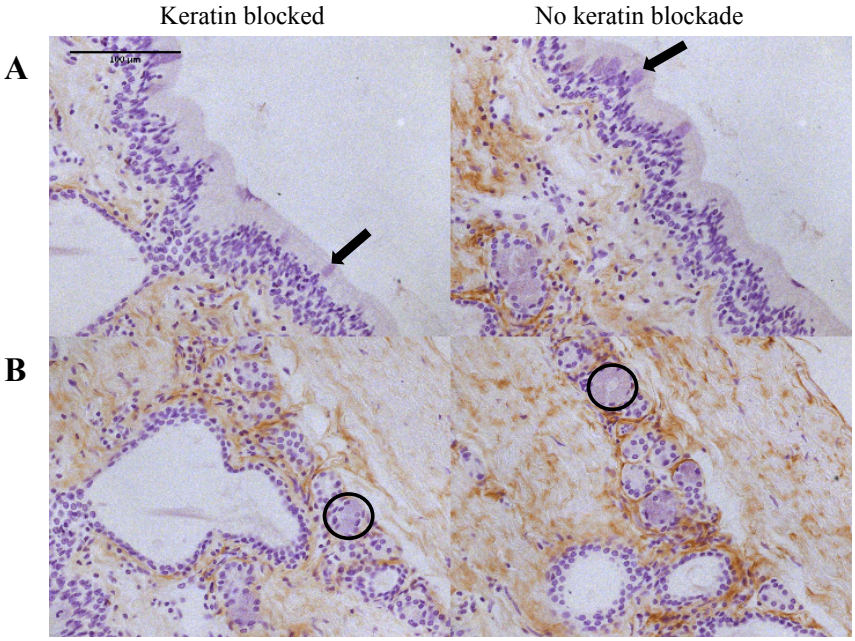
We first considered that a simple solution to the issue of excessive background Muc5ac-2 staining of the ethanol-fixed tissue (without the antigen-retrieval step) would be to use the antibody more dilute than the 1:100 dilution we had been employing. We carried out an experiment staining serial sections of ethanol-fixed equine respiratory tissue from the trachea of a randomly selected healthy abattoir horse (AH5) with a variety of Muc5ac-2 dilutions in block as follows: 1:100, 1:250, 1:500, 1:1000 (*Fig. 4.10*). Increasing the antibody dilution did reduce the DAB positive background staining, but unfortunately concurrently led to reduced positive mucin cell staining, in an incremental manner (*Fig. 4.10*). The resultant mucin cell staining with dilutions other than 1:100 for the Muc5ac-2 antibody were considered not satisfactory to be able to discern all mucin-containing tissue for the project and so we decided to investigate another way of reducing the background staining whilst still using the 1:100 antibody: block dilution.

Fig. 4.10 **DILUTION SERIES FOR MUC5AC-2 IMMUNOHISTOCHEMISTRY DETECTION IN EQUINE RESPIRATORY TISSUE**. Slides made from serial sections of ethanol-fixed paraffin-embedded equine respiratory tracheal epithelium from one randomly selected healthy abattoir horse (AH5) were stained using Muc5ac-2 purified antibody at 1:100, 1:250, 1:500 and 1:1000 dilutions, incubated overnight and detected using DAB colour detection without antigen retrieval step. Haematoxylin counterstain. **A**. Epithelium **B**. Sub-mucosal glands. Red arrows indicate positive DAB stained epithelial goblet cells. Black arrows indicate negative DAB stained epithelial goblet cells. Red circles indicate positive DAB stained sub-mucosal glands. Black circles indicate negative DAB stained sub-mucosal glands. Original magnification x 40. Scale bar 100 µm. Representative images shown.



We considered that we might have anti-keratin antibodies in our polyclonal antiserum, which could be the reason for our strong DAB positive background staining in our slides. In order to try to block keratin-antibody binding, we tried first incubating the Muc5ac-2 antibody with equine keratin before performing DAB detection. We used leftover saliva previously collected from healthy horses (for use in a different experiment) as our source of equine keratin. Serial sections of ethanol-fixed mid-tracheal tissue from a randomly selected healthy abattoir horse (AH3) were incubated with Muc5ac-2 antibody (1:100 dilution) with or without simultaneous incubation with equine saliva (1:100 dilution of saliva in antibody in block). The immunohistochemical protocol with DAB staining was then followed, omitting the antigen-retrieval step (refer to *Methods*; *Fig. 4.11*).

Fig. 4.11 **KERATIN BLOCKADE TO ELIMINATE BACKGROUND DAB STAINING: MUC5AC-2 IMMUNOHISTOCHEMISTRY DETECTION IN EQUINE RESPIRATORY TISSUE**. Slides made from ethanol-fixed paraffin-embedded equine respiratory mid-tracheal epithelium from one randomly selected healthy abattoir horse (AH3), were stained using Muc5ac-2 purified antibody at 1:100 dilution incubated overnight and detected using DAB colour detection without antigen retrieval step with or without addition of saliva (1:100) to antibody incubation step to block keratin binding cross-reactivity. Haematoxylin counterstain. **A**. Epithelium. **B.** Sub-mucosa. Black arrows indicate negative DAB stained epithelial goblet cells. Black circles indicate negative DAB stained sub-mucosal glands. Original magnification x 40. Scale bar 100 µm. Representative images shown.



The results of this experiment were inconclusive, as inexplicably, there was no DAB positive staining of mucin cells, and also no clear difference in background staining in either the keratin-blocked or unblocked slides (*Fig. 4.11*). The experiment was repeated again, this time using a number of randomly selected tracheal slides cut from both ethanol and anatomically paired formalin-fixed tissues from the same (AH3) and 2 additional healthy abattoir horses (AH1, AH8) to ensure that it was not simply that the first horse chosen did not have any Muc5ac present in the cells examined. The antibody dilution used remained at 1:100, and ethanol-fixed tissue did not undergo the antigen-retrieval step whilst formalin fixed tissue did (refer to *Methods*). Again the results were inconclusive with a lack of much Muc5ac-positive DAB staining of mucin cells and no discernable difference in background DAB staining between keratin blocked and non-blocked tissues (*Appendix II: Fig. AII.4*).

The lack of a definite Muc5ac-specific antibody for use in immunohistochemistry ultimately lead to our discontinuation of attempts to stain respiratory sections for Muc5ac. Although we would have ideally liked to have included data on Muc5ac in the project, we had already established that Muc5b is the main equine airway secreted polymeric mucin in both health and RAO (refer to *Chapter 3*), and so we concentrated on Muc5b and general mucin stain (PAS AB) for the remainder of this chapter.

In the following experiments we examine the anatomical distribution of mucin producing cells in equine airway epithelium of healthy abattoir horses. We examine slides stained with general mucin stain PAS AB and also focus on and compare to staining of serial-cut slides for the mucin Muc5b, using the refined Muc5b immunohistochemistry protocol outlined above.

4.2.2 HEALTHY HORSE AIRWAY MUCIN PRODUCING CELLS

In order to investigate the anatomical location of mucin producing cells in the healthy horse airway we used respiratory tracts collected from 9 healthy abattoir horses (AH1 – AH9) immediately post mortem. We used identical areas of each respiratory tract from each horse, collected and fixed in ethanol and processed to form wax blocks for cutting sections for mucin identification (refer to *Methods*). This included identical areas of the dorsal, ventral, left and right cranial, middle and caudal trachea as well as a section

of the primary, secondary and tertiary bronchi from each horse (refer to *Methods*). Two serial slides were cut from each block (15 blocks for each horse) and one stained with the general mucin stain (PAS AB) and the other DAB stained immunohistochemically for Muc5b using the refined protocol outlined in *4.2.1.4*, (involving incubation of the purified Muc5b-5 antibody at 1:100 dilution in block overnight and processing omitting the antigen retrieval step and then counterstaining with haematoxylin).

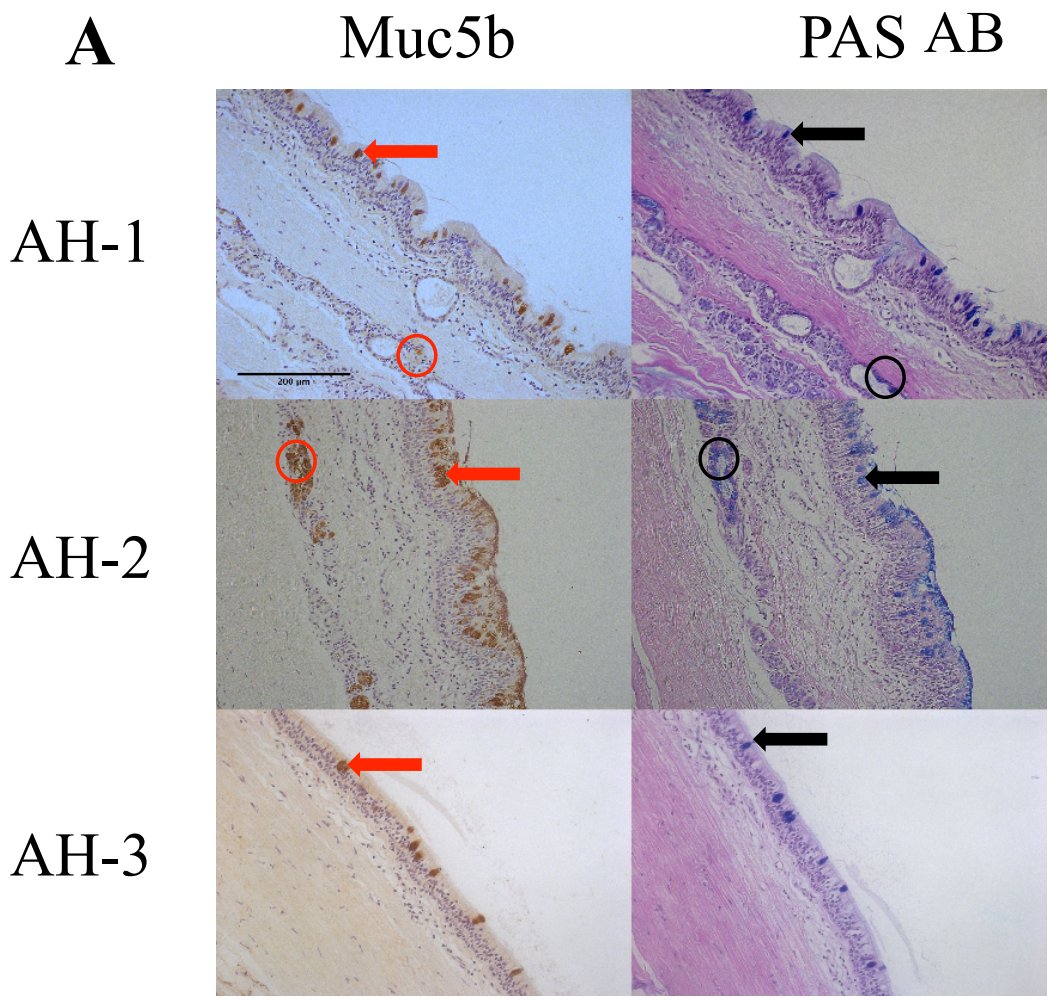
Three x 20 magnification images of epithelium and sub-mucosa for analysis of mucin-cell staining were obtained from each slide for both the PAS AB and Muc5b staining methods. Measurements of goblet cell (GC) number, epithelium basement membrane length, sub-mucosal gland total and stain-positive size and areas were recorded for each image and the mean calculated for each slide (refer to *Methods*). From these measurements, calculations were performed to produce the following parameters for each anatomical area:

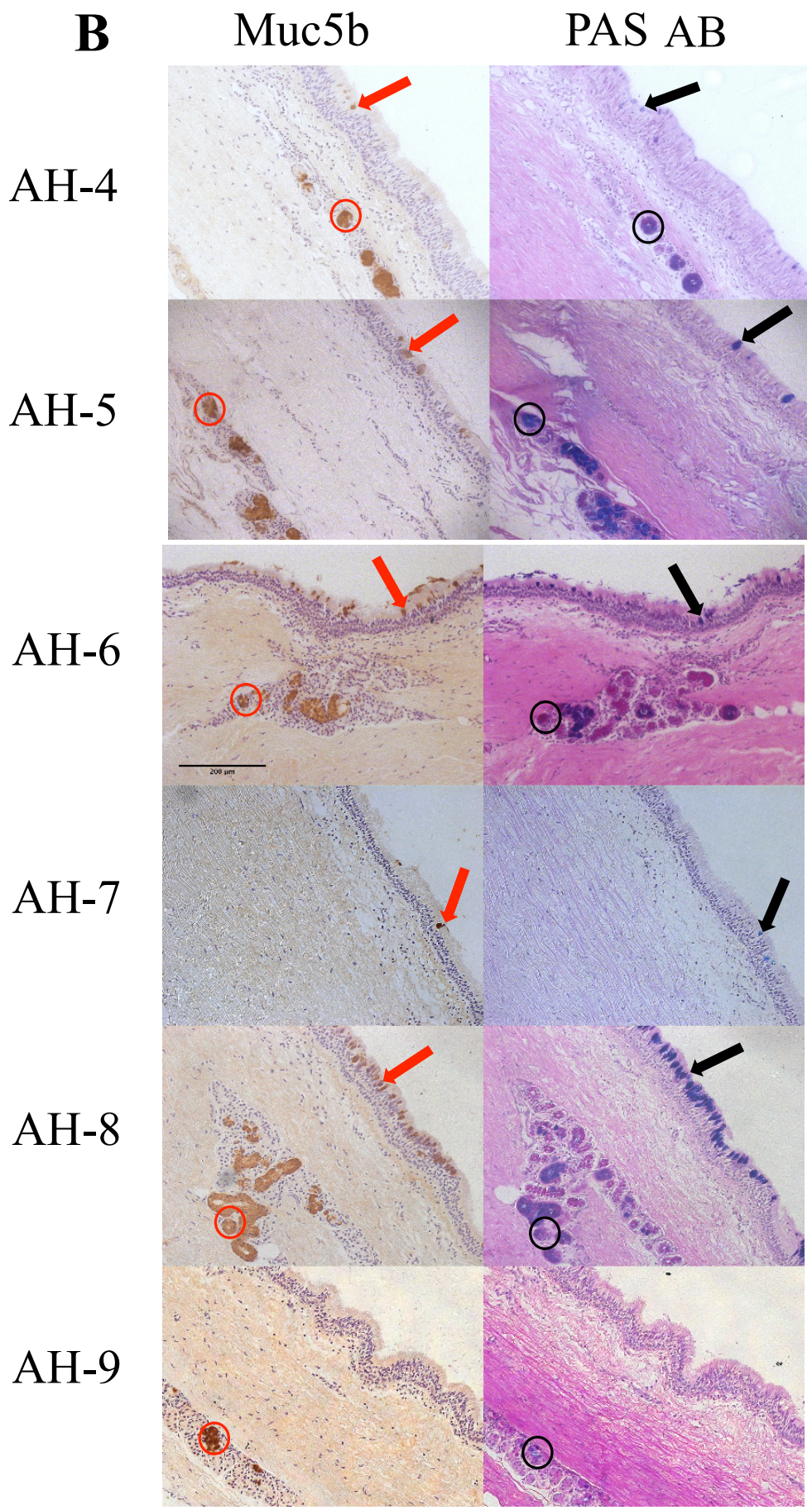
A. Number of GC per 1000 μm of epithelium
B. Mean GC size (μm^2)
C. Percentage of sub-mucosal gland mucin-stain positive
D. Area of sub-mucosal gland mucin-stain positive (μm^2)

Fig.4.12 shows paired representative x 20 magnification images for identical anatomical location tracheal epithelium (right mid trachea) for both staining methods, Muc5b and PAS/AB, for each horse AH1 – AH9. The data gathered from all of the slides were analysed at the image (3 per slide), slide (mean of 3 images) and horse level. The calculated parameters for mean goblet cell number, size and sub-mucosal gland positive stain area size and percentage of the sub-mucosal gland staining positive were first evaluated for overall differences between horses (inter-horse variation), and differences between Muc5b and PAS AB staining in each individual horse (intra-horse variation). As previously mentioned, we were interested for the purpose of future work on biopsies of epithelium, in any differences in anatomical distribution of goblet cells along the respiratory tract, especially as earlier researchers have pointed to fewer of epithelial goblet cells and sub-mucosal glands in the dorsal trachea (Pirie *et al.*, 1990a; Widdicombe and Pecson, 2002). We therefore then went on to examine the data for significant differences at different anatomical locations, namely any differences between the tracheal and bronchial parameters, cranial vs. middle vs. caudal trachea, around the circumference of

the trachea (dorsal vs. ventral vs. left vs. right), and finally any differences between the primary, secondary and tertiary bronchi.

Fig. 4.12 **MUC5B AND PAIRED PAS AB STAINING OF HEALTHY HORSE RESPIRATORY EPITHELIUM**. Slides made from ethanol-fixed paraffin-embedded equine respiratory tracheal epithelium from 9 healthy abattoir horses (AH-1 to AH-9), were stained using Muc5b purified antibody at 1:100 dilution incubated overnight and detected using DAB colour detection with the antigen retrieval step omitted (left-hand side images). Serial section slides were stained with general mucin stain PAS AB (right hand-side images). Counterstaining with haematoxylin. **A**. AH1 – AH3; **B**. AH4 – AH9. Representative images of identical anatomical location from each horse right mid-trachea epithelium. Red arrows indicate Muc5b positive epithelial goblet cells. Red circles indicate Muc5b positive sub-mucosal gland. Black arrows indicate PAS AB positive epithelial goblet cells. Black circles indicate PAS AB positive sub-mucosal gland. Original magnification x 20. Scale bar 200 µm.




B
Muc5b
PAS AB
AH-4
AH-5
AH-6
AH-7
AH-8
AH-9

4.2.2.1 INTER- AND INTRA-HORSE VARIATION: HEALTHY ABATTOIR HORSES

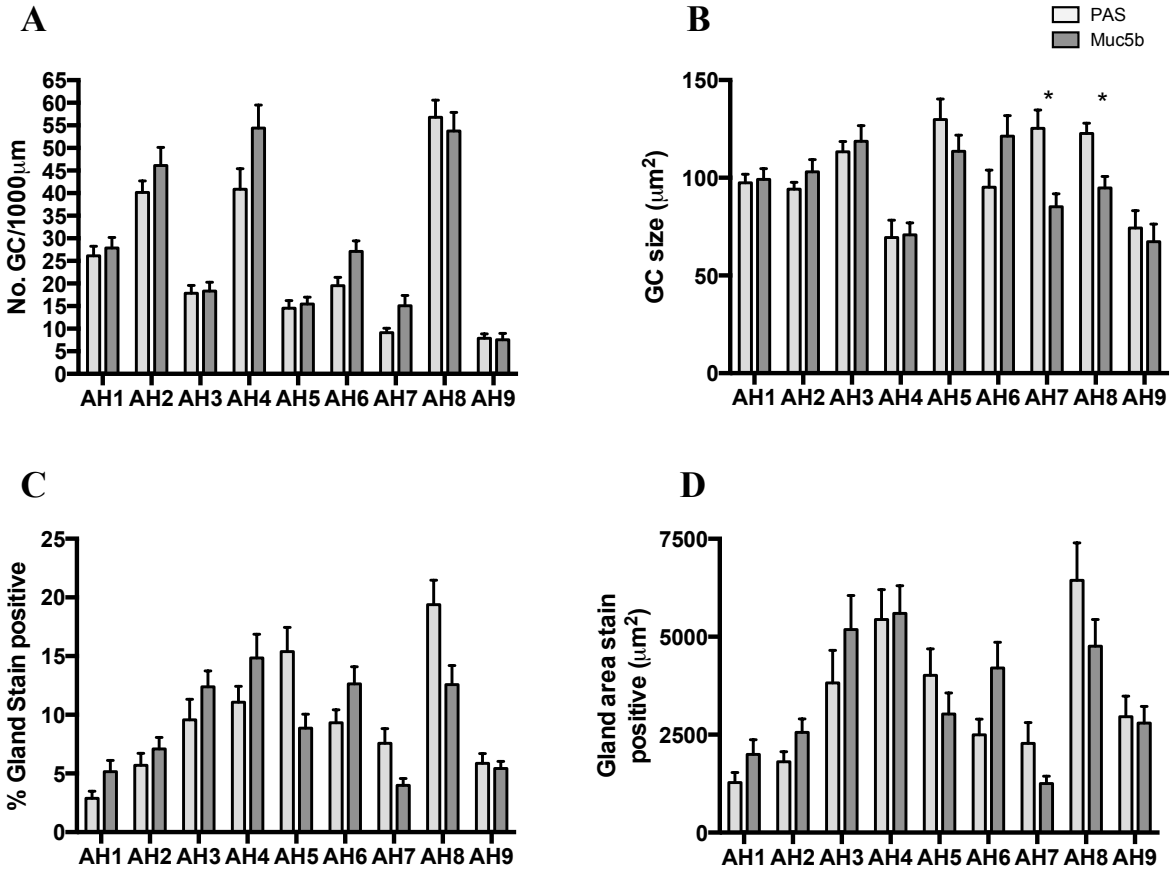
At the horse level, by considering the 4 calculated parameters (A - D outlined in 4.2.2) for all slides from each horse (45 images per horse: 15 slides, 3 images per slide), we examined overall differences between horses (inter-horse variation) and between the Muc5b and PAS AB staining in each individual horse (intra-horse variation). The purpose of this was to assess what variation in goblet cell number and size, and sub-mucosal gland mucin-cell size and percentage of gland containing mucin exists, if any, between healthy horses. It was also important to establish how closely related the parameters for Muc5b and PAS AB staining were, to gauge how much of the overall mucin stain (PAS AB) could be attributed to Muc5b, rather than Muc5ac or any other mucin.

We used grouped analysis multiple t-tests to examine differences within each individual horse (intra-horse) between PAS AB and Muc5b for the four calculated parameters (A-D), based on all data collected from all images. There was no significant difference ($P > 0.05$) between the Muc5b and PAS AB staining for any horse for goblet cell number, percentage of gland stain positive or size of sub-mucosal gland staining positive (*Fig. 4.13*). There was a significantly larger ($P < 0.05$) mean size of goblet cells with PAS AB stain than Muc5b for 2 horses (AH7 and AH8), with there being no significant difference in goblet cell size between the 2 staining methods for the remaining 7 horses. Considering the 4 calculated parameters overall, there was very little difference between measurements for the 2 staining methods, inferring that most cells that are PAS AB positive are also Muc5b positive, and that there are very few PAS AB positive Muc5b negative cells. Those cells that are PAS AB positive and Muc5b negative could potentially be Muc5ac positive; we do not know if the Muc5b positive PAS AB positive cells are also Muc5ac positive. For the following analysis of inter-horse variation, we consider the PAS AB and Muc5b data together for each horse, given that we found very little difference in the calculated parameters between staining methods.

In grouped two-way ANOVA analysis of the horse level data with multiple comparisons, there is a significant variation of GC number per 1000 μm epithelium between horses (7.5 to 56.8 goblet cells per 1000 μm epithelium; $P < 0.0001$) (*Fig. 4.13 A*). There were many significant differences comparing each horse to every other horse, the only non-significant differences ($P > 0.05$) being between AH1 vs AH6; AH2 vs AH4; AH3 vs AH5, 6, 7; AH4 vs AH8; AH5 vs AH6, 7, 9; and AH7 vs AH9 (*Fig. 4.13 A*). Abattoir horses AH2, 4 and 8 had higher mean numbers (40.1 to 56.8) of goblet cells per

1000 µm epithelium, while horses AH3, 5, 7 and 9 had a mean of less than 20 goblet cells per 1000 µm epithelium for both staining methods (Muc5b and PAS AB).

Fig. 4.13 **INDIVIDUAL HORSE DATA FROM PAS AB AND MUC5B STAINING OF RESPIRATORY TISSUES FROM HEALTHY HORSES**. Tissue sections of 15 identical anatomical locations along the trachea and bronchi from 9 healthy abattoir horses (AH1 - 9), were cut in serial section and stained for PAS AB and Muc5b. For each slide, 3 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following mean parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for each horse is shown using multiple t-tests to assess PAS AB vs Muc5b staining intra-horse variation. * indicates significant difference ($P < 0.05$) between PAS AB and Muc5b staining for individual horses. Letters "AH" followed by number refer to individual abattoir horses. PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +SEM plotted.



There was a significant variation in mean GC size between horses (67.3 μm^2 to 129.8 μm^2; $P < 0.0001$) (*Fig.4.13 B*). However the significance arises from 2 horses (AH4, AH9) which are similar to each other but significantly had smaller goblet cell size (67.3 to 74.3 μm^2) compared to the 7 other horses (AH1, 2, 3, 5, 6, 7, 8), and there is no significant difference in mean GC size between those other seven horses (85.2 to 129.8 μm^2) (*Fig. 4.13 B*). We wondered if these two horses has correspondingly more goblet cells in compensation for their smaller goblet cell staining, however when we examined this we found that AH4 was at the higher end of the GC number spectrum (40.9 per 1000 μm epithelium), whilst that of AH9 was the lowest number, with a mean of 7.9 GC per 1000 μm epithelium. We then looked at the data from all of the horses to investigate if there was any correlation of GC size with number, but found that the mean number of goblet cells per length of epithelium does not correlate with mean goblet cell size (Spearman's rank correlation $r = 0.62$, $P > 0.05$, data not shown).

There was a significant inter-horse variation in the mean percentage (2.9 % to 19.4 %; $P < 0.0001$) of sub-mucosal gland staining positive for mucin on grouped two-way ANOVA with multiple comparisons of the horse level data (*Fig. 4.13 C*). Specifically, there were significant inter-horse differences ($P < 0.05$) in percentage of gland staining mucin positive between AH3 vs AH1, 2, 7; AH4 vs AH1, 2, 5, 6, 7, 9; AH5 vs AH1; and AH8 vs AH1, 2, 5, 6, 7, and 9 (*Fig. 4.13 C*).

Grouped two-way ANOVA with multiple comparisons analysis of the horse level data showed a significant variation between horses of the mean size of mucosal gland staining positive for PAS AB/ Muc5b (1252 to 6441 μm^2; $P < 0.0001$) (*Fig. 4.13 D*). There was a wide variation between horses with non-significant differences ($P > 0.05$) between the following horses: AH1 vs AH2, 7, 9; AH2 vs AH7, 9; AH3 vs AH4, 5, 6; AH4 vs AH5, 6, 8; AH5 vs AH 6, 8; AH7 vs AH9 (*Fig. 4.13 D*). We investigated if the area, or size, of the gland staining positive for mucin (Muc5b or PAS AB) correlated to the percentage of the gland area that stained positive for mucin, to be able to tell if mucin-positive staining increases with an increased total sub-mucosal gland size (which includes non-mucin producing tissue). The area of positive staining gland and the percentage of gland staining positive correlated significantly for all horses (Spearman's rank correlation, $r = 0.93$, $P < 0.001$, data not shown), indicating a larger gland contains an increased percentage of mucin-positive tissue.

We wanted to identify if the amount of mucin-positive staining in the epithelium had any relationship to the amount of mucin-positive staining in the sub-mucosal glands,

such as a positive or negative correlation of number or size of goblet cells with amount of mucin in the deeper glands. We examined if there was any correlation of GC size or number with either sub-mucosal gland mucin-positive area or percentage, but found no correlation for any of these comparisons (data not shown).

From the above data, we now know that, considering the tracheal and bronchial epithelium as a whole, there can be large variation of goblet cell number between horses, whilst goblet cell size is, in general, less varied between individual healthy horses. We also know that the percentage and size area of mucin-positive sub-mucosal gland varies greatly between individual horses and that there is a positive correlation between the size of the gland area containing mucin and the percentage of the total gland area containing mucin. This tells us that in glands with a greater mucin-positive area size, the proportion of the gland containing mucin is larger, rather than just the glands are overall larger in those individuals. There was no correlation between epithelial goblet cell and sub-mucosal gland positive mucin staining in these 9 healthy horse airways.

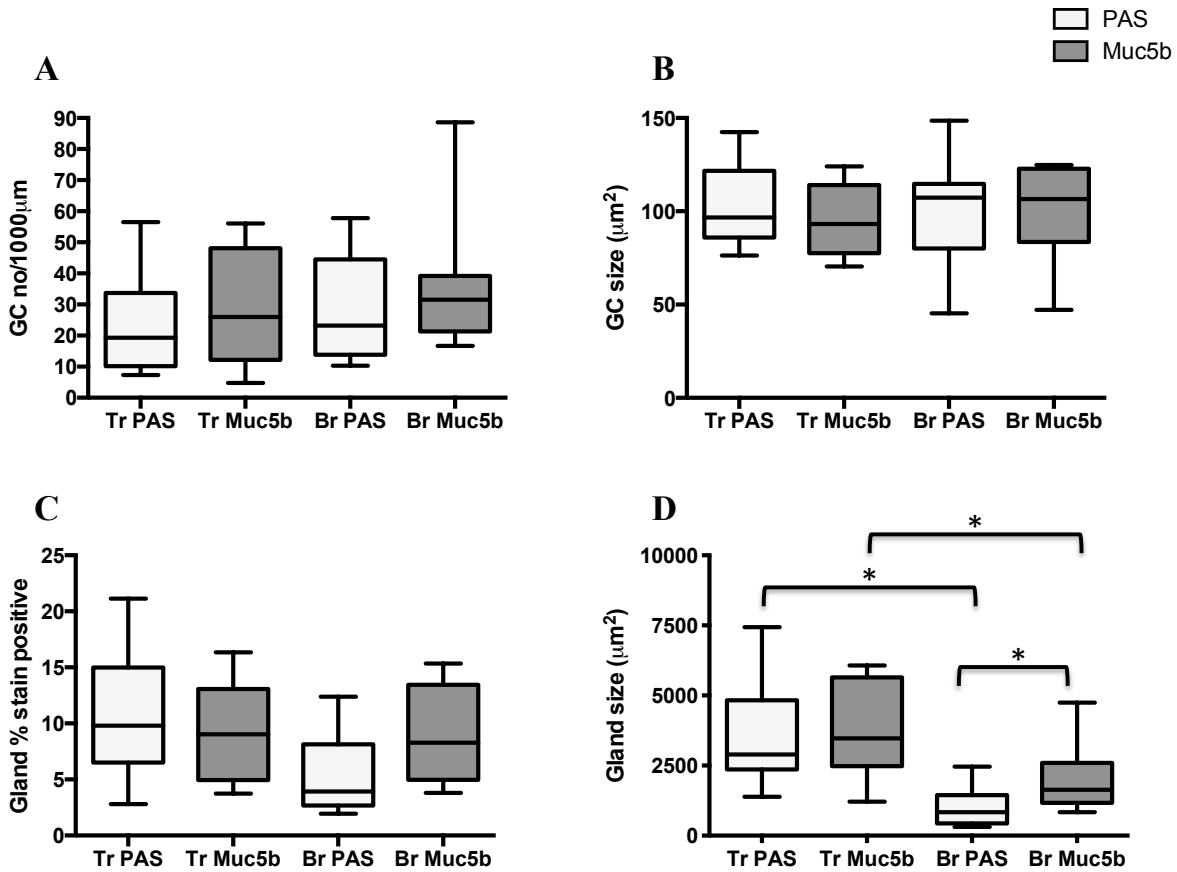
We have established that Muc5b and general (PAS AB) mucin staining are not significantly different in each horse when considering mean values taken from data across all sections of the respiratory tract (cranial, middle, caudal trachea and primary to tertiary bronchi). It is also important to establish if there is variation anatomically throughout the respiratory tract at different anatomical locations for the mucin producing cells. We examine this in the following section by comparing Muc5b and PAS staining overall tracheal to bronchial values for each of the parameters A-D, by looking at the cross-sectional area of the trachea (dorsal vs ventral vs left vs right), by comparing the cranial, middle and caudal sections of the trachea, and finally by comparing the primary to secondary and tertiary level bronchi. We examined all of the data at the image, slide and horse level.

4.2.2.2 TRACHEA VS BRONCHI

We examined the Muc5b and PAS AB staining data (calculated parameters A-D, refer to *4.2.2*) for tracheal sections grouped together and compared it to the data for the bronchial sections grouped together, at the image, slide and horse level. There was no significant difference at any level in the number or size of goblet cells, or the Muc5b

versus PAS stain method measurements, between the trachea and bronchi of these healthy horses (Mann-Whitney U, P > 0.05) (*Fig. 4.14 A, B*).

Fig. 4.14 **TRACHEA VS BRONCHI DATA FROM PAS AB AND MUC5B STAINING OF RESPIRATORY TISSUES FROM HEALTHY HORSES**. Tissue sections of 15 identical anatomical locations along the trachea and bronchi from 9 healthy abattoir horses were cut in serial section and stained for PAS AB and Muc5b. For each slide, 3 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters for each anatomical area (trachea, bronchi): **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Paired data (Tr vs Br for PAS AB and Muc5b separately, Tr PAS AB vs Tr Muc5b, and Br PAS AB vs Br Muc5b) were analysed at image, slide and horse level by Mann-Whitney U t-tests. Horse level data shown (* indicates significant difference, P < 0.05). PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +/-SEM plotted. Tr = trachea, Br = bronchi. GC = goblet cell.



The percentage of tracheal sub-mucosal gland staining mucin positive compared to the bronchial glands was significantly larger ($P < 0.05$) for Muc5b at the image level, and for PAS at the image and slide level, but not significant ($P > 0.05$) for either stain method at the horse level (Mann-Whitney U) (*Fig. 4.14 C*). The percentage of Muc5b positive gland staining was significantly more than that for PAS AB staining of the bronchial glands at image level (Mann-Whitney U, $P < 0.05$) (*Fig. 4.14 C*); however there was no other significant difference found between PAS AB and Muc5b percentage staining of either tracheal or bronchial sub-mucosal glands at any level. Mucosal glands in the trachea had a larger positive gland stain size (for both PAS and Muc5b) compared to those of the bronchi, at all 3 levels (image, slide and horse) (Mann-Whitney U, $P < 0.05$) (*Fig. 4.14 D*). The area of Muc5b gland-positive stain size was found to be significantly larger than that for PAS for the bronchial sub-mucosal glands (Mann-Whitney U, $P < 0.05$) (*Fig. 4.14 D*).

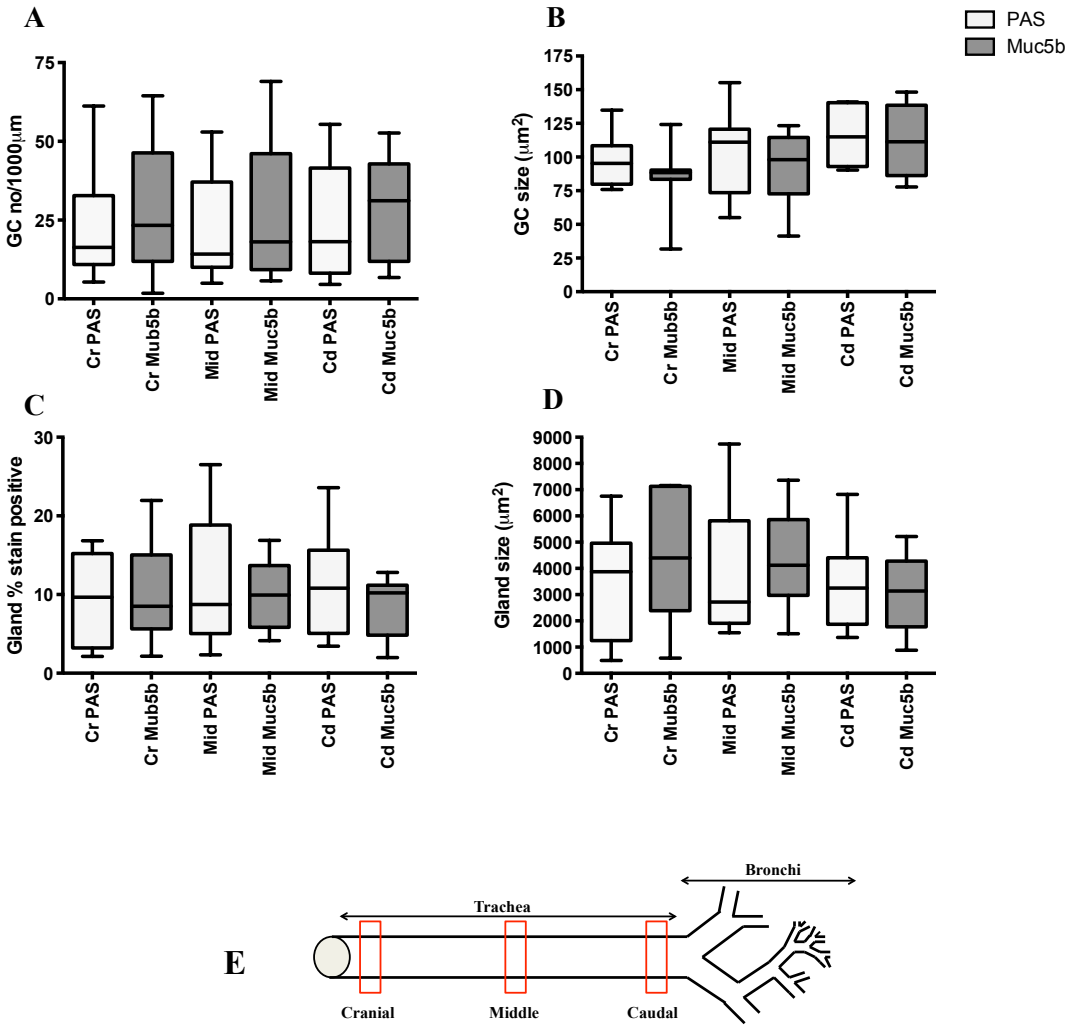
The above information tells us that goblet cell size and number in the epithelium does not differ between the trachea and first 3 generations of bronchi in the healthy horse. The amount of mucin in the tracheal sub-mucosal glands is more abundant, having a larger area than in the bronchial glands. The percentage of sub-mucosal gland that stains positive for mucin is greater in the trachea than the bronchi to a degree, though not at all levels. This tells us that the overall gland size in the trachea is larger than the bronchi, as might be expected, whilst the amount of gland area occupied by mucin-positive tissue does not necessarily alter overall.

We have established the differences in goblet cell size and number and sub-mucosal gland size that exist between the trachea and the bronchi. What we still need to investigate is if there are more specific variances along the length and around the circumference of the trachea, and through the generations of bronchi, which we do in the following sections.

4.2.2.3 LENGTH OF TRACHEA: CRANIAL VS MIDDLE VS CAUDAL

We examined data for PAS AB and Muc5b staining calculated parameters A - D, at the image, slide and horse level, from 3 points along the length of the trachea, namely cranial, middle and caudal tracheal sections (refer to *Methods*). There was no significant difference between the PAS or Muc5b staining methods for any measurement (one-way ANOVA Kruskall-Wallis with Dunn's comparison post test, $P > 0.05$)(*Fig. 4.15*).

Fig. 4.15 **TRACHEA CRANIAL, MIDDLE AND CAUDAL SECTION: DATA FROM PAS AB AND MUC5B STAINING OF RESPIRATORY TISSUES FROM HEALTHY HORSES**. Tracheal tissue sections of 3 identical anatomical locations, (cranial, middle and caudal trachea), from 9 healthy abattoir horses, were cut in serial section and stained for PAS AB and Muc5b. For each slide, three x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters for each anatomical area: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (µm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (µm^2). Grouped data (Cr vs Mid vs Cd for PAS AB and Muc5b separately, Cr PAS AB vs Cr Muc5b, Mid PAS AB vs Mid Muc5b, and Cd PAS AB vs Cd Muc5b) was analysed at image, slide and horse level by one-way ANOVA Kruskall-Wallis test with Dunn's post comparison. Horse level data shown (* indicates significant difference, $P < 0.05$). PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +/-SEM plotted. **E**. Schematic diagram representing areas of interest in graphs (not to scale). Cr = cranial, Mid = middle, Cd = caudal.



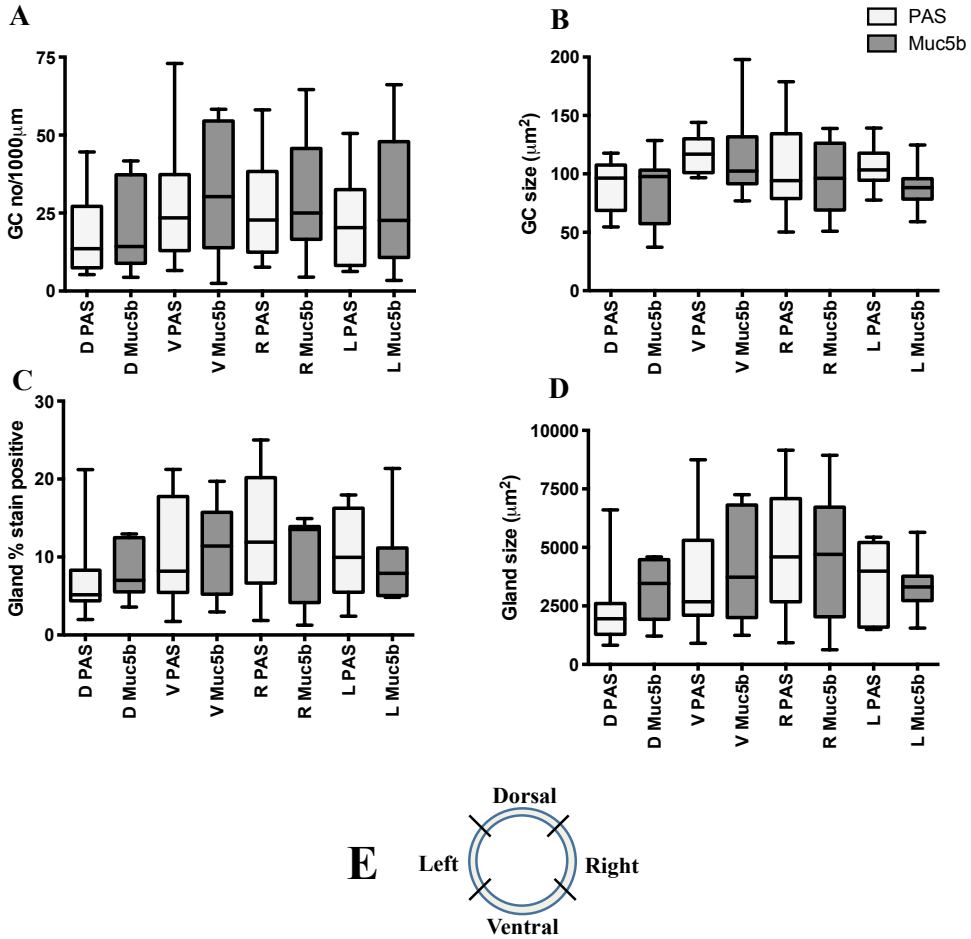
There was no significant difference in mucin producing goblet cell number or size, or gland area or percentage staining positive, at the 3 different measurement points along the length of the trachea (Kruskall-Wallis with Dunn's comparison post test) (*Fig. 4.15*). So we now know that the number and size of epithelial goblet cells does not vary in distribution along the length of the trachea, nor does the size and amount of mucin in the sub-mucosal glands along the length of the trachea. Previously, the dorsal trachea has been identified as having fewer goblet cells and smaller sub-mucosal glands than the ventral trachea (Pirie *et al.*, 1990a; Widdicombe and Pecson, 2002), so we wanted to confirm this by comparing the 4 parameters in dorsal, ventral, left and right circumferential sections of trachea from all 3 sampled sections of trachea (cranial, middle and caudal), which we do in the following section.

4.2.2.4 CIRCUMFERENCE OF TRACHEA: DORSAL VS VENTRAL VS RIGHT VS LEFT

We examined data for PAS AB and Muc5b staining calculated parameters A-D, at the image, slide and horse level, from the 3 sections along the length of the trachea, (cranial, middle and caudal) grouping data from all 3 sections according which part of the tracheal circumference they were from, i.e. dorsal, ventral, left and right (refer to *Methods*). Examining the data from the 4 areas of the circumference of the trachea, there was no significant difference between the PAS or Muc5b staining methods for any measurement (*Fig. 4.16*). There was no difference in epithelial goblet cell number or size, or sub-mucosal gland area or percentage staining positive, for any of the four measurement points (A - D) around the cross-sectional circumference of the trachea ($P > 0.05$, Kruskall-Wallis with Dunn's comparison post test) (*Fig. 4.16*).

The above information tells us that the size and number of goblet cells and sub-mucosal glands does not significantly alter at any point of the cranial, middle or caudal portion of the trachea or at any point around its circumference. We previously identified some differences between the trachea and the bronchi (refer to *4.2.2.2*) and so for the next section we examine the bronchi data more closely.

Fig. 4.16 **TRACHEA DORSAL, VENTRAL LEFT AND RIGHT SECTIONS: DATA FROM PAS AB AND MUC5B STAINING OF RESPIRATORY TISSUES FROM HEALTHY HORSES**. Tracheal tissue sections of 4 cross-sectional locations, (dorsal, ventral, left and right), at 3 identical anatomical locations along the length of the trachea (cranial, middle and caudal), from 9 healthy abattoir horses, were cut in serial section and stained for PAS AB and Muc5b. For each slide, three x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters for each cross-sectional area (Dorsal, ventral, left and right): **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped data (D vs V vs L vs R for PAS AB and Muc5b separately, D PAS AB vs D Muc5b, V PAS AB vs V Muc5b, L PAS AB vs L Muc5b, and R PAS AB vs R Muc5b) was analysed at image, slide and horse level by one-way ANOVA Kruskall-Wallis test with Dunn's post comparison. Horse level data shown (no significant differences noted). PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +/-SEM plotted. **E**. Schematic diagram representing areas of interest in graphs. Not to scale. D = dorsal, V = ventral, R = right, L = left.



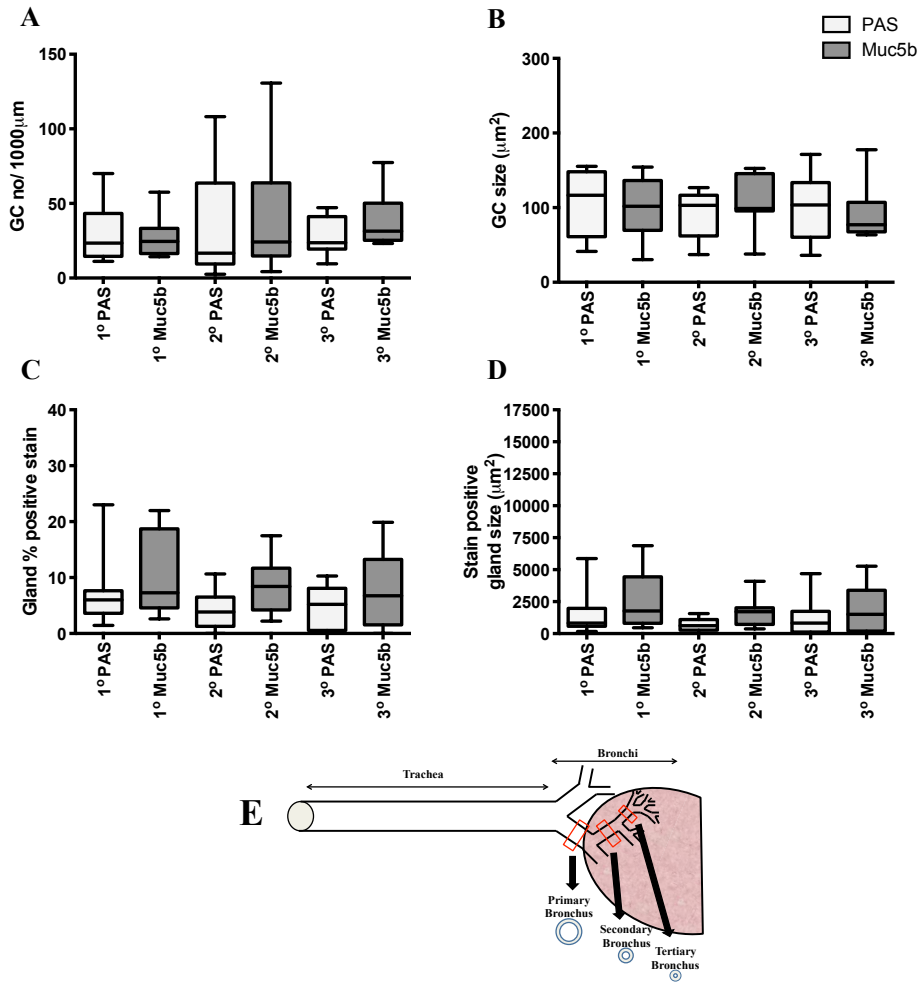
4.2.2.5 BRONCHI: PRIMARY VS SECONDARY VS TERTIARY

We examined data for PAS AB and Muc5b staining calculated parameters A - D, at the image, slide and horse level, grouping the 3 sizes of bronchi, (primary, secondary and tertiary) separately and comparing them (refer to *Methods*). The data from the primary, secondary and tertiary bronchi did not reveal any significant differences between the PAS or Muc5b staining methods for any of the calculated measurements A - D (*Fig. 4.17*). The percentage of gland staining positive was higher but not statistically significant for Muc5b compared to PAS AB for all 3 sizes of bronchi (Kruskall-Wallis with Dunn's comparison post test) (*Fig. 4.17*). There was no significant difference in mucin producing goblet cell number or size, or gland area or percentage staining positive, for any of the 3 bronchi areas (primary, secondary or tertiary) examined (Kruskall-Wallis with Dunn's comparison post test) (*Fig 4.17*).

From the preceding experiments we have established that, in the healthy horse airway, Muc5b staining very closely resembles general mucin (PAS AB) staining; that there is great variation in both goblet cell number and mucin-positive gland size and percentage staining between individual horses, but that goblet cell size is less varied between horses. We have learnt that although there is great variation between horses, that within each horse there is very little variation along the length of the trachea or around its circumference, or between the different sizes of bronchi. There is more mucin in the sub-mucosal glands of the trachea than those in the bronchi. We have also learnt that the bigger the sub-mucosal gland size, the greater percentage of the gland contains mucin.

This information all relates to healthy horses and so in the next section we go on to examine data gathered form RAO diseased horses and controls, to see what differences there are, if any, in mucin-producing cells of the respiratory tract of horses suffering from RAO. We have seen previously (refer to *Chapter 3*) that RAO horses have increased tracheal mucus compared to controls, whether housed in a disease-inducing "exposed" environment or kept outdoors in a "not-exposed" environment. Therefore we were interested to know if the distribution, size and number of mucin-producing cells changes in RAO horses when they are housed in exposed or not-exposed environmental conditions, and whether or not there was also any change in control horses.

Fig. 4.17 **PRIMARY SECONDARY AND TERTIARY BRONCHI: DATA FROM PAS AB AND MUC5B STAINING OF RESPIRATORY TISSUES FROM HEALTHY HORSES**. Tissue sections from the primary, secondary and tertiary bronchi of 9 healthy abattoir horses, were cut in serial section and stained for PAS AB and Muc5b. For each slide, three x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters for each anatomical area (Cranial, middle and caudal trachea): **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped data (1^0 vs 2^0 vs 3^0 for PAS AB and Muc5b separately, 1^0 PAS AB vs 1^0 Muc5b, 2^0 PAS AB vs 2^0 Muc5b, and 3^0 PAS AB vs 3^0 Muc5b) was analysed at image and horse (= same as slide level) level by one-way ANOVA Kruskall-Wallis test with Dunn's post comparison. Horse level data shown (no significant difference noted). PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +/-SEM plotted. **E**. Schematic diagram representing areas of interest in graphs. Not to scale. 1^0 = primary bronchi, 2^0 = secondary bronchi, 3^0 = tertiary bronchi.



4.2.3 RAO AND HEALTHY CONTROL HORSES: AIRWAY MUCIN PRODUCING CELLS FROM HORSES IN EXPOSED AND NOT-EXPOSED ENVIRONMENTS

To permit us to investigate the distribution of mucin-producing cells in the RAO-diseased horse airway under different environmental conditions, and compare those findings to healthy horses under the same conditions, we were generously given access to a tissue bank (Tb) of respiratory tissue from RAO and healthy control horses (n = 19). The respiratory tissue had been collected immediately post mortem from pre-determined anatomical locations, namely a portion of caudal trachea (T), and a portion of mid lung (L), thus allowing us to examine the epithelium and sub-mucosa of tracheal sections and bronchial epithelium (refer to *Methods*). The tissue bank samples had been collected immediately post-mortem from horses that were healthy controls (n = 8: Tb-1 to Tb-8) or RAO-diseased (n = 11, Tb-9 to Tb-19), housed in a dusty stable "exposed" environment (causing clinically apparent disease in the RAO-affected horses but not the healthy controls) or outside in a "not-exposed" environment prior to euthanasia. The horses had all had lung function testing performed prior to euthanasia to confirm RAO or control status (refer to *Appendix II: Fig. AII.5*). At the time of creation of the tissue bank, samples were fixed in formalin and processed to produce wax-embedded tissue blocks for histological slide preparation. Tissue blocks from both anatomical locations, T and L, were not unfortunately available from all horses. We have already established above (refer to *4.2.2*) that the distribution of mucin-producing cells is not different at different anatomical locations of the trachea; therefore we assume that the data from the T caudal tracheal section are representative of the whole trachea.

Slides were cut from all available blocks stained with the general mucin stain (PAS AB) and counterstained with haematoxylin. We also cut serial slides from all blocks and stained immunohistochemically for Muc5b using the refined protocol outlined above (refer to *4.2.1*) for formalin-fixed tissue (1:100 dilution of purified antibody Muc5b-5 in block with an antigen retrieval step). As discussed previously, the background staining and variable positive staining of mucin tissue when formalin-fixed tissue is used is not ideal for immunohistochemical identification of Muc5b; however we only had access to formalin-fixed tissues for this experiment and so we examine the data available to us. In the following section we discuss the results we obtained for the general mucin stain and the Muc5b immunohistochemistry. We captured x 20 magnification images of as much

epithelium and sub-mucosa as was present on each slide, leading to a variation of number of images per slide (n = 1 - 9).

Measurements made from the images captured were the same as those made in the earlier healthy abattoir horse study (refer to *Methods* and *4.2.2*). From these measurements, calculations were again performed to produce the following parameters A - D for each x 20 magnification image:

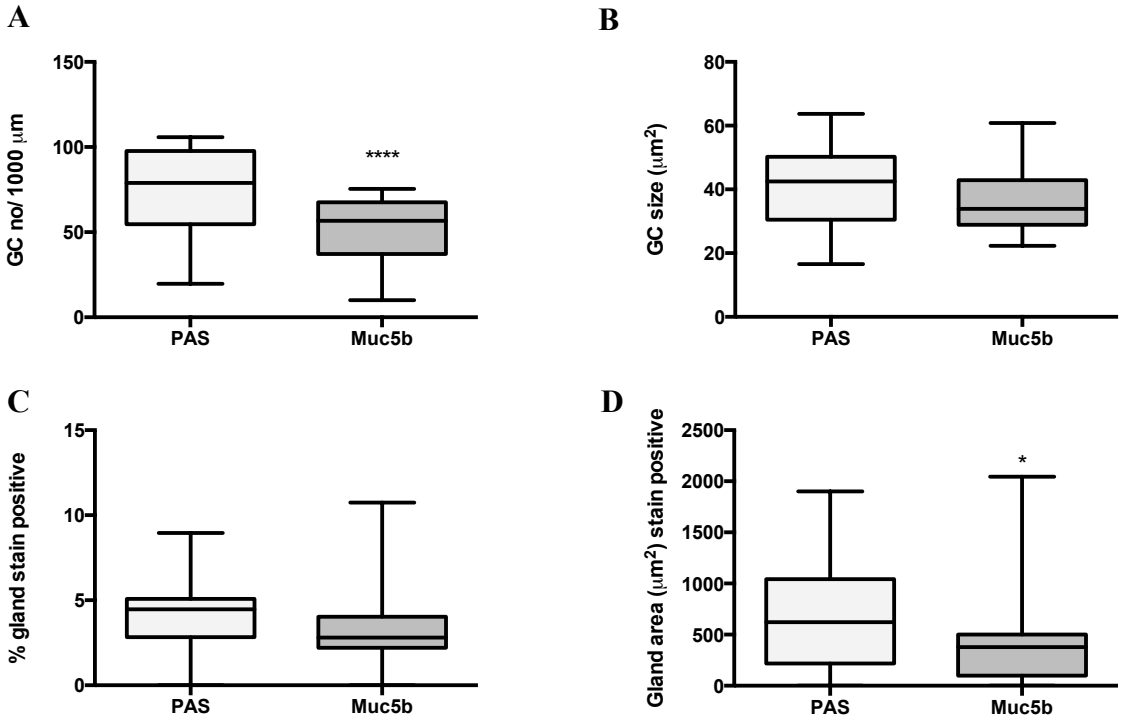
A. Number of GC per 1000 μm of epithelium
B. Mean GC size (μm^2)
C. Percentage of sub-mucosal gland mucin-stain positive
D. Area of sub-mucosal gland mucin-stain positive (μm^2)

The mean data for each slide was calculated based on the data obtained from all of the images from each slide. We examined all of the data (all mean slide data from T and L sections together for each horse) to assess for any differences in the 4 calculated parameters between each individual horse (inter-horse variation), between the groups RAO horses and control horses, between the sub-groups of "exposed" and "not-exposed" horses within each group (RAO or control horse groups) and comparing across groups (RAO vs control). We also examined the data from the separate anatomical areas L and T for the above considerations. There were no gland data available for Tb-12 as no sub-mucosal glands were present in the fixed tissue on the slides. In tissues where glands were visualised but no positive mucin staining was observed, a value of 0 was assigned to the gland data for that image.

4.2.3.1 STAINING VARIATION: GENERAL MUCIN STAIN VERSUS Muc5b

In the study of healthy horses we had noted very little difference in the calculated parameters A - D between the general mucin stain (PAS AB) and the Muc5b immunohistochemistry. The tissues used in this RAO vs control horse tissue bank study had been fixed in formalin, as opposed to the ethanol fixed tissues used in the healthy abattoir horse study, and we had previously discovered that Muc5b staining using our purified antibody was not as good as positive identification of mucin tissue as with ethanol-fixed tissue samples. Therefore we firstly assessed if there were any significant differences between the 2 staining methods for the 4 calculated parameters using a Wilcoxon matched-pairs signed rank test (using the mean data for each slide) (*Fig. 4.18*).

Fig. 4.18 **PAS AB AND MUC5B DATA FROM STAINING OF RESPIRATORY TISSUES FROM CONTROL AND RAO HORSE TISSUE BANK**. Tissue sections from 19 horses (8 healthy control horses 11 RAO-diseased horses) from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections, were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for each staining method is shown with Wilcoxon paired signed-rank tests assessing overall PAS AB vs Muc5b staining for the calculated parameters A - D. *P < 0.05 **** P <0.0001 between PAS AB and Muc5b staining methods. PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +/-SEM plotted.



We found that there was no significant difference (P > 0.05) in mean goblet cell size between the 2 staining methods, but that there were significantly fewer goblet cells identified using the Muc5b (P < 0.0001) compared to the PAS AB stain (*Fig. 4.18*). There was no difference in gland percentage staining positive for mucin between the 2 staining methods (P > 0.05), however the area of gland staining positive for mucin was less with Muc5b than the general mucin stain (P < 0.05) (*Fig.4.18*). The differences identified here between the Muc5b and PAS AB staining methods could be due to the poorer

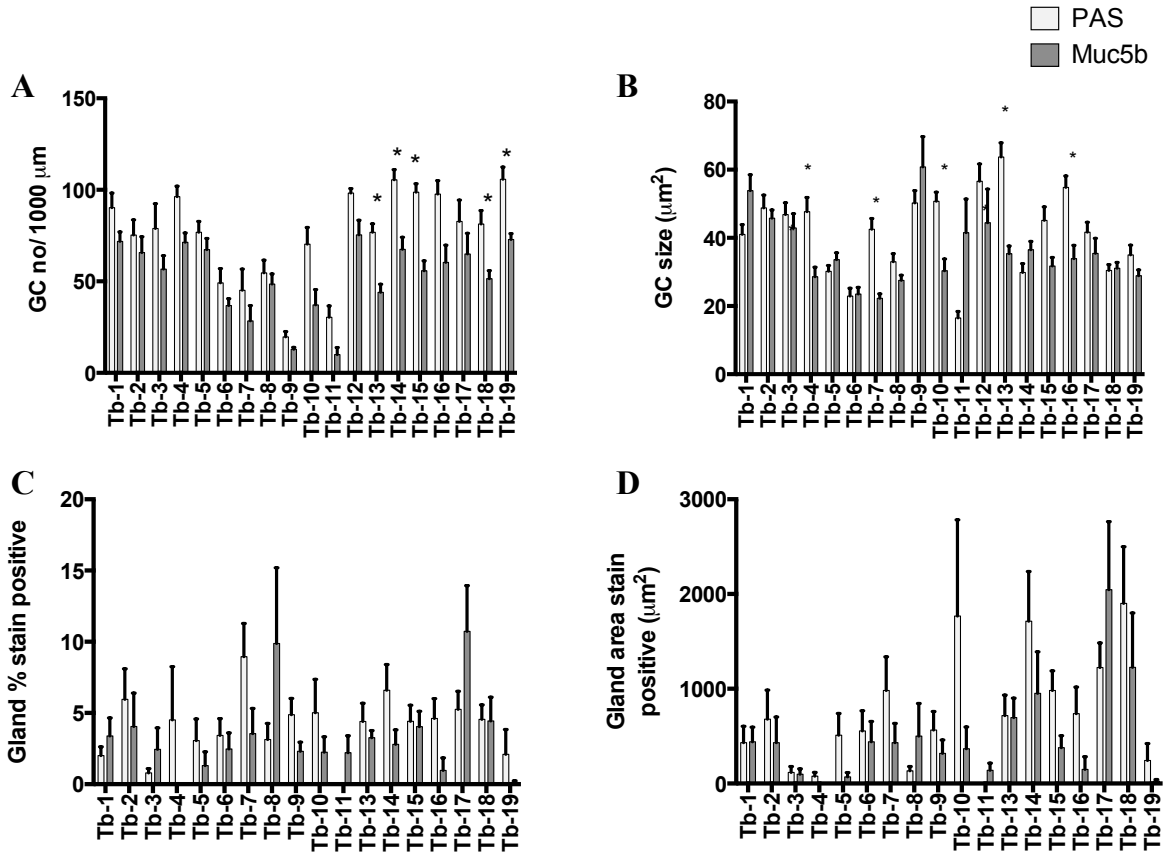
identification of mucin tissue using Muc5b on formalin-fixed tissue, or because some mucin in the mucin-tissue was another mucin, such as Muc5ac, or a combination of both of these factors. We next went on to assess differences in the 4 calculated parameters between each individual horse.

4.2.3.2 INTER- AND INTRA-HORSE VARIATION: RAO AND HEALTHY CONTROL HORSES (Tb-1 to Tb-19).

We used grouped analysis multiple t-tests to examine differences within each individual horse (intra-horse) between PAS AB and Muc5b for the four calculated parameters (A - D), based on all data collected from all images (*Fig. 4.19*). There was a significantly higher number of goblet cells per 1000 μm epithelium ($P < 0.05$) with PAS AB stain than for Muc5b for 5 RAO horses (Tb-13, 14, 15, 18, 19), with there being no significant difference in goblet cell number between the 2 staining methods for the remaining RAO and all healthy control horses (*Fig. 4.19A*). There was a significantly larger mean GC size ($P < 0.05$) with PAS AB stain than for Muc5b for 5 horses (control horses Tb-4, 7; RAO horses 10, 13, 16), with there being no significant difference in mean goblet cell size between the 2 staining methods for the remaining RAO and healthy control horses (*Fig. 4.19B*). Due to the differences found between the PAS AB and Muc5b staining methods, we considered each staining method separately for the analysis of variation between horses.

In grouped two-way ANOVA analysis of the horse level data with multiple comparisons, there is a significant variation of GC number per 1000 μm epithelium between horses ($P < 0.0001$) (*Fig. 4.19A*). For the general mucin stain there were significant differences comparing each horse to every other horse, ($P < 0.05$) with fewer goblet cells in control horse Tb-6 vs control horses Tb-1,4, and vs RAO horses Tb-14,16,18,19; control horse Tb-7 vs control horses Tb-1,4, and RAO horses Tb-12 to Tb-19; control horse Tb-8 vs RAO horses Tb-14,15,19; RAO horse Tb-9 vs all horses except control horses Tb-6 to Tb-8, and RAO horse Tb-11; and RAO horse Tb-11 vs control horses Tb-1,3,4,5, and RAO horses Tb-12 to Tb-19 (*Fig. 4.19A*). For the Muc5b stain, control horse Tb-7, and RAO horses Tb-9 and Tb-11 had a significantly lower number of GC than other RAO and control horses (Tb-1, 4, 5, 14 for Tb-7; Tb-1 to 5, 12, 14 to 19 for Tb-9; and Tb-1 to 5, 12, 14 to 19 for Tb11).

Fig. 4.19 **INDIVIDUAL HORSE DATA FROM MUCIN STAINING OF RESPIRATORY TISSUES FROM CONTROL AND RAO HORSE TISSUE BANK**. Formalin-fixed caudal trachea and mid lung tissue sections from each of 8 healthy control horses (Tb-1 to Tb-8) and 11 RAO-diseased horses (Tb-9 to Tb-19), were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for each horse is shown using multiple t-tests to assess PAS AB vs Muc5b staining intra-horse variation. * indicates significant difference ($P < 0.05$) between PAS AB and Muc5b staining for individual horses. Letters "Tb" followed by number refer to individual tissue bank horses. PAS AB light grey bars, Muc5b dark grey bars. Horse were kept in either exposed or not-exposed controlled environments prior to euthanasia (control horses not-exposed Tb-1 and Tb-2, exposed Tb-3 to Tb-8; RAO-diseased horses not exposed Tb-9 to Tb-13, exposed Tb-14 to Tb-19) No gland data available for Tb-12. Graphs show mean +SEM plotted.



There is a significant variation in mean goblet cell size between horses in grouped two-way ANOVA analysis of the horse level data with multiple comparisons ($P < 0.0001$) (*Fig.4.19 B*). For PAS AB data, control horses Tb-5 and Tb-6 and RAO horses have significantly ($P < 0.05$) smaller goblet cells than other control and RAO horses; whilst RAO horse Tb-13 has significantly larger goblet cells than numerous other RAO and control RAO horses (*Fig. 4.19 B*). For the Muc5b data, control horse Tb-1 and RAO horse Tb-9 have significantly ($P < 0.05$) larger goblet cells than other control and RAO horses; whilst control horses Tb-6 and Tb-7 have significantly smaller ($P < 0.05$) goblet cells than other control and RAO horses (*Fig. 4.19 B*).

There was a significant inter-horse variation in the mean percentage of sub-mucosal gland staining positive for mucin on grouped two-way ANOVA with multiple comparisons of the horse level data specifically, for RAO horse Tb-18 vs control horses Tb-3 and Tb-4 for PAS AB staining, and between RAO horse Tb-17 and control horses Tb-4 and Tb-5 for Muc5b staining (*Fig. 4.19 C*). Grouped two-way ANOVA with multiple comparisons analysis of the horse level data showed a significant variation between horses of the mean size of mucosal gland staining positive for the PAS stain between control horses Tb-3 and Tb-7 only; and for Muc5b staining between RAO horse Tb-17 and some other control (Tb-3, 6) and RAO (Tb-13, 14, 16, 19) horses (*Fig. 4.19 D*).

We looked at the data from all of the horses to investigate if there was any correlation of GC size with GC number for each staining method. We did not find a correlation of the mean number of goblet cells per length of epithelium with mean goblet cell size (Spearman's rank correlation $r < 0.22$, $P > 0.05$, data not shown). This lack of positive correlation is the same as we had seen previously in the study of healthy abattoir horses.

As we had previously identified a positive correlation with the area, or size, of the gland staining positive for mucin (Muc5b/ PAS AB) with the percentage of the gland area that stained positive for mucin, we investigated any correlation for the data from all tissue bank horses. There was again a positive correlation of the area of positive staining gland and the percentage of gland staining positive for both staining methods (Spearman's rank correlation, $r > 0.75$, $P < 0.0004$, data not shown), indicating a larger gland contains an increased percentage of mucin-positive tissue.

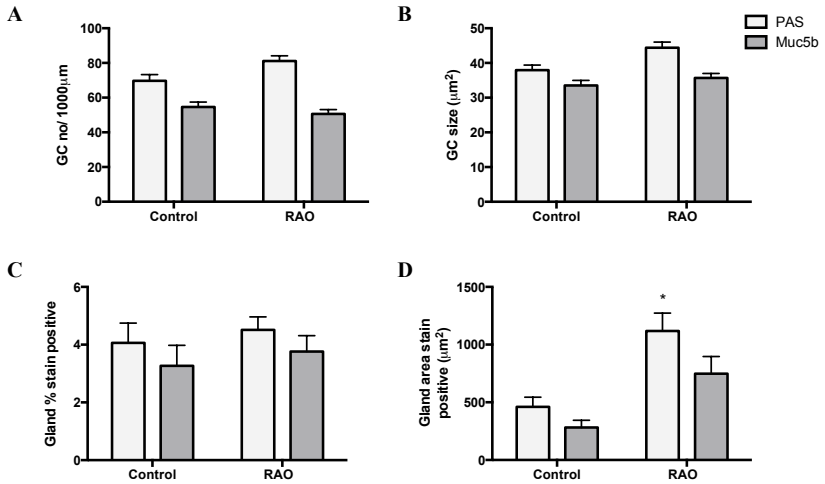
From the information gathered above, we now know that there is a difference in the 4 calculated parameters between individual horses, both control and RAO, in the following

section we go on to examine the data for differences between the exposed and not-exposed horses and then also between different anatomical areas (bronchi (L) vs trachea (T)).

4.2.3.3 RAO HORSE GROUP VERSUS CONTROL HORSE GROUP

We grouped the data for control horses and the data for RAO horses separately and analysed the 4 calculated parameters for both staining methods separately using a 2-sided unpaired t-test (*Fig. 4.20*). The mean size of gland staining positive for mucin was significantly ($P < 0.05$) larger for RAO horses compared to control horses, for the PAS data (*Fig. 4.20*). There were no other significant findings between the RAO and control group horse parameters when the data were analysed in this way. The RAO and control group horses could further be split into sub-groups dependent on exposure status at time of euthanasia (exposed/ not-exposed), and so we examine differences between the subgroups in the following section.

Fig. 4.20 **RAO HORSE GROUP VERSUS CONTROL HORSE GROUP COMPARISON OF STAINING METHODS.** Tissue sections from 19 horses (8 healthy control horses, 11 RAO-diseased horses) from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections, were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Analysis of mean data for each group of horses for each staining method is shown with a 2-sided unpaired t-test for the calculated parameters A - D. *$P < 0.05$ between control and RAO horse groups. PAS AB light grey bars, Muc5b dark grey bars. Graphs show mean +SEM plotted.



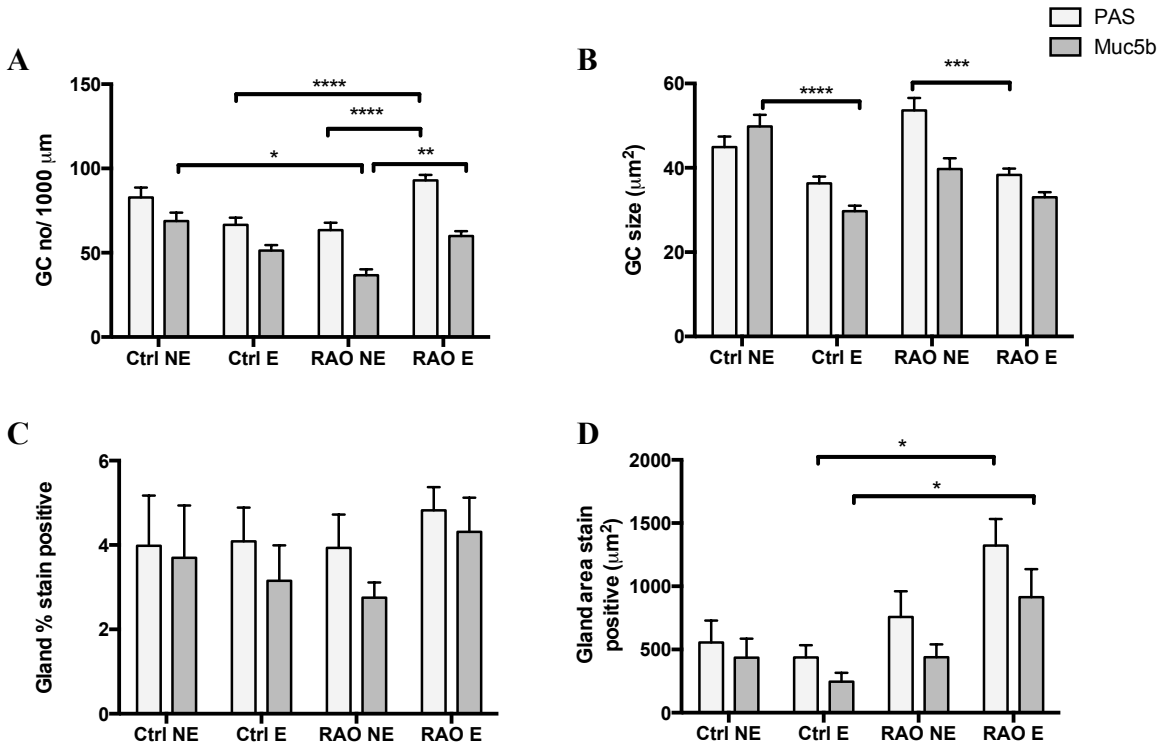
4.2.3.4. RAO HORSE VERSUS CONTROL HORSE SUB-GROUPS: EXPOSED VERSUS NOT-EXPOSED ENVIRONMENT

Analysis of the 4 calculated parameters for the 2 staining methods using Kruskall-Wallis ANOVA with multiple comparisons was performed for the control and RAO horses split into sub-groups for being housed in an exposed or not-exposed environment prior to euthanasia (*Fig. 4.21*). There were significantly more goblet cells per length of epithelium for the RAO exposed sub-group compared to the control exposed sub-group ($P < 0.0001$) for PAS staining. The RAO exposed subgroup also had a significantly larger number of goblet cells per 1000 µm epithelium than the RAO not-exposed subgroup for both staining methods ($P < 0.01$) (*Fig. 4.21 A*). There were significantly more goblet cells per 1000 µm epithelium for the control not-exposed subgroup of horses compared to the RAO not-exposed group ($P < 0.05$) (*Fig.4.21 A*). Mean goblet cell size was significantly larger ($P < 0.001$) for the control not-exposed versus control-exposed subgroup for Muc5b staining, and for the RAO not-exposed versus the RAO exposed sub-groups with PAS staining (*Fig. 4.21 B*). Analysis of the gland data for the sub-groups revealed significantly larger gland area staining mucin-positive for the RAO exposed versus the control exposed groups for both staining methods ($P < 0.05$) (*Fig. 4.21 C*). There was no significant difference between subgroups for the percentage of gland staining mucin-positive (*Fig. 4.21 D*).

This information tells us that exposed RAO horses have larger areas of mucin-positive sub-mucosal gland than healthy control horses when both are housed in an exposed dusty stable environment. The mean number of goblet cells in RAO-exposed horses is higher than those in both RAO not-exposed horses and control-exposed horses. The mean number of goblet cells staining positive for Muc5b in RAO not-exposed horses is fewer than those in control not-exposed horses. It also tells us that for both control and RAO diseased horses, goblet cells are larger when horses are housed in a not-exposed clean air environment than when housed in an exposed dusty stable environment.

We had previously identified in healthy abattoir horses that there is no significant difference between the number and size of epithelial goblet cells between the trachea and bronchi, but that the sub-mucosal glands of the trachea contain a larger amount of mucin than those in the bronchi. In the following section we analyse these data for the RAO and control exposed and not-exposed groups of horses. Unfortunately there was no tracheal tissue available in the tissue bank from control not-exposed horses.

Fig. 4.21 **GROUPED CONTROL AND RAO HORSE GROUPS: EXPOSED AND NOT-EXPOSED SUB-GROUPS DATA FROM MUCIN STAINING OF RESPIRATORY TISSUES**. Tissue sections from each of 8 healthy control horses and 11 RAO-diseased horses from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for RAO and control horses divided into exposed and not-exposed subgroups (according to controlled environmental housing conditions prior to euthanasia) is shown using Kruskall-Wallis ANOVA multiple group comparisons to assess inter-group variation in the parameters A - D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. PAS AB light grey bars, Muc5b dark grey bars. RAO and control (Ctrl) horses were kept in either exposed (E) or not-exposed (NE) controlled environments prior to euthanasia. Graphs show mean +SEM.



4.2.3.5 RAO HORSE VERSUS CONTROL HORSES: SEPARATE ANATOMICAL AREAS TRACHEA AND MID-LUNG BRONCHI FOR EXPOSED VERSUS NOT-EXPOSED ENVIRONMENT SUB-GROUPS

We used grouped analysis multiple t-tests to examine differences within each sub-divided sub-group of horses between PAS AB and Muc5b staining for the four calculated parameters (A - D), based on mean data collected from all images for each horse (refer to *Appendix II: Fig. AII.5*). In agreement with our previous findings of whole group data, there was a significantly higher number of goblet cells per 1000 μm epithelium ($P < 0.05$) with PAS AB stain than for Muc5b for some sub-groups of horses. Specifically, this was for 3 anatomical area sub-groups of horses, namely the control exposed mid-lung bronchi group, the RAO not-exposed mid-lung bronchi group, and the RAO exposed trachea group. There was a significantly larger mean goblet cell size ($P < 0.05$) for PAS AB stain than for Muc5b for the RAO horse not-exposed tracheal area sub-group only (refer to *Appendix II: Fig. AII.5*). There was no significant difference between the 2 staining methods for the sub-mucosal gland data (refer to *Appendix II: Fig. AII.5*).

Analysis of the 4 calculated parameters for the 2 staining methods using Kruskall-Wallis ANOVA with multiple comparisons was performed for the control and RAO horses split into sub-groups for being housed in an exposed or not-exposed environment at the time of euthanasia and further split according to anatomical area of tissue bank sections, namely caudal trachea (T) and mid-lung bronchi (L) (*Figs. 4.22, 4.23, Appendix II: Fig AII.2, Table AII.1*). As we have already mentioned any differences between the PAS AB and Muc5b staining above, we will consider the data analysis for the 2 staining methods separately here, to make it simpler to explain.

For the general mucin stain (PAS AB) data, there were significantly more goblet cells per 1000 μm epithelium for the control not-exposed L sub-group compared to the RAO not-exposed L sub-group ($P < 0.05$) (*Fig. 4.22 A*). The control exposed L subgroup had significantly more GC per 1000 μm epithelium than the control exposed T subgroup ($P < 0.0001$), and significantly fewer ($P < 0.05$) than their RAO counterpart sub-group. The exposed control horse T subgroup had significantly fewer GC per 1000 μm epithelium than their RAO counterparts. Within the RAO horse group subgroups, there were significantly more GC per 1000 μm epithelium in the RAO exposed L subgroup than the not-exposed L or exposed T subgroups ($P < 0.0001$). The RAO not exposed T subgroup had significantly fewer ($P < 0.05$) GC per 1000 μm epithelium than their exposed counterparts (*Fig. 4.22 A*).

Fig. 4.22 **GROUPED CONTROL AND RAO HORSE EXPOSED AND NOT-EXPOSED SUB-GROUPS DIVIDED INTO ANATOMICAL AREAS: TRACHEA VS LUNG. DATA FROM PAS AB STAINING OF RESPIRATORY TISSUES**. Tissue sections from each of 8 healthy control horses and 11 RAO-diseased horses from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for RAO and control horses divided into exposed and not-exposed subgroups (according to controlled environmental housing conditions at time of euthanasia) further subdivided into information for separate anatomical areas, trachea (T) and mid-lung bronchi (L) is shown using Kruskall-Wallis ANOVA multiple group comparisons to assess inter-group variation in the parameters A - D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. PAS AB light grey bars. RAO and control (Ctrl) horses were kept in either exposed (E) or not-exposed (NE) controlled environments prior to euthanasia. Graphs show mean +SEM.

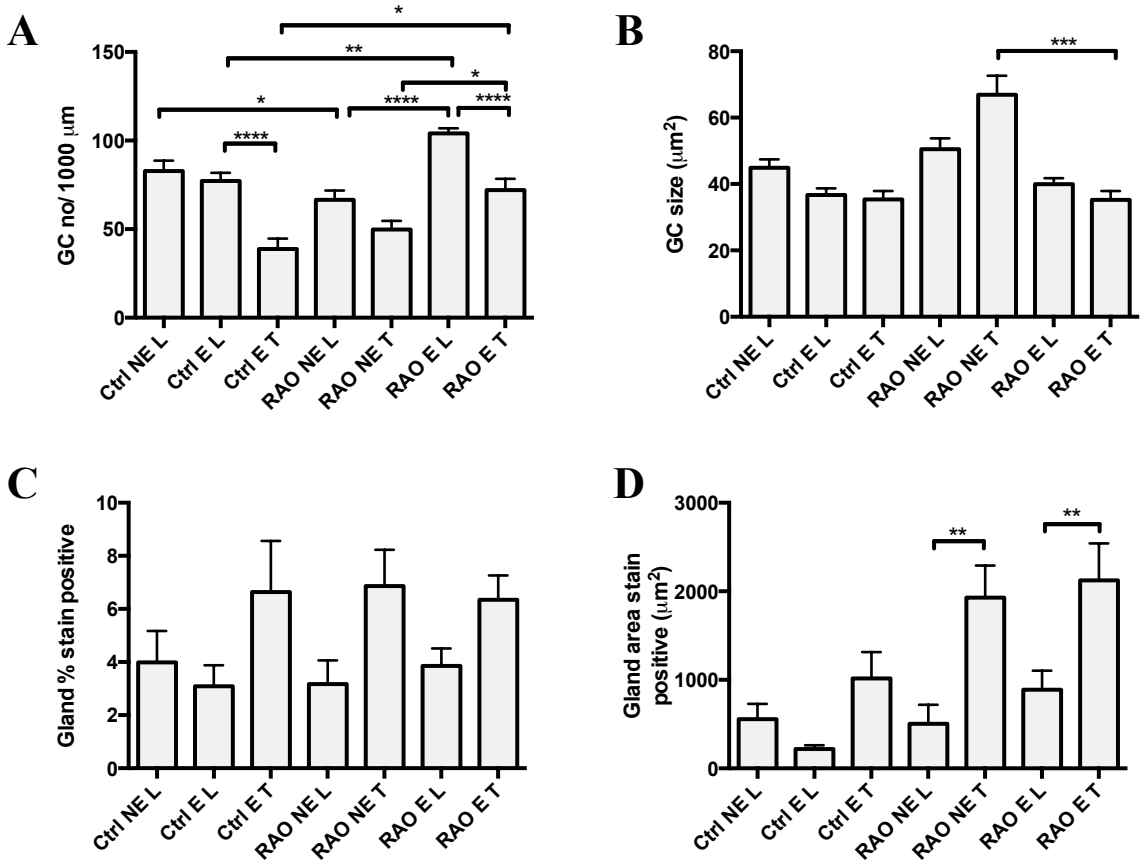
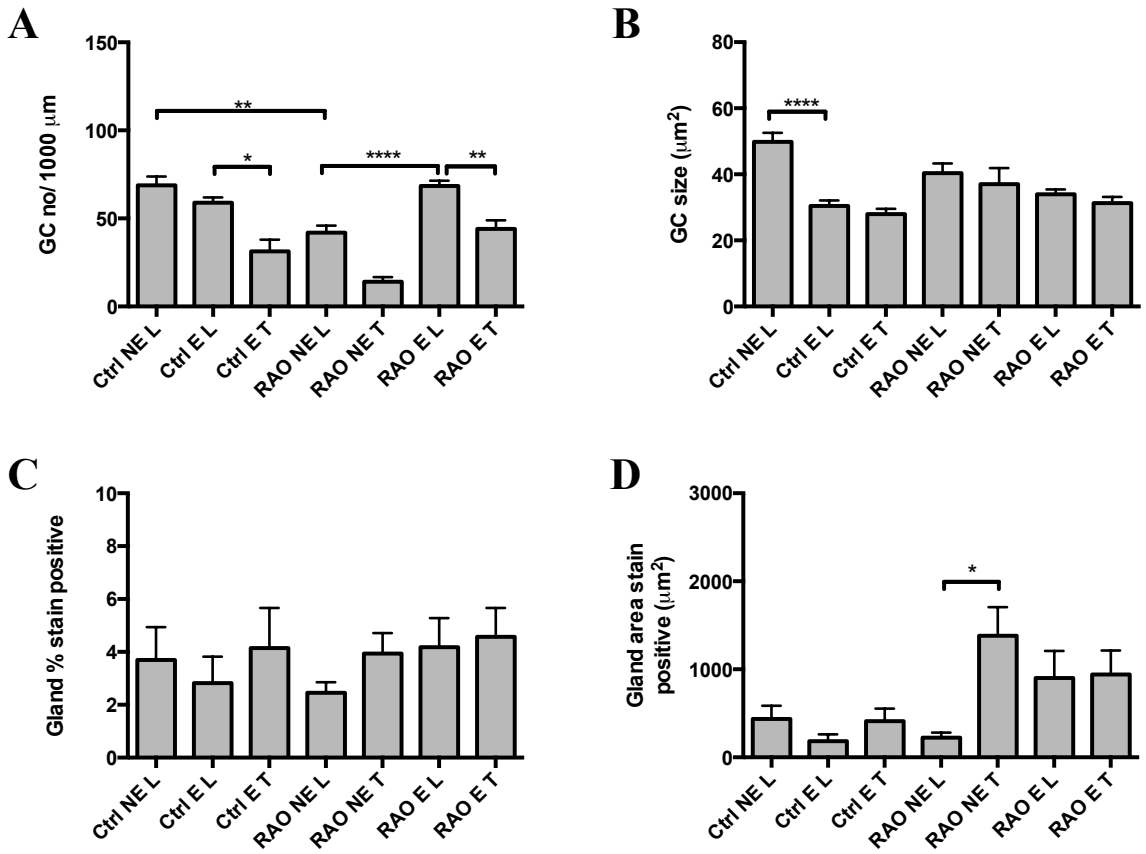


Fig. 4.23 **GROUPED CONTROL AND RAO HORSE EXPOSED AND NOT-EXPOSED SUB-GROUPS DIVIDED INTO ANATOMICAL AREAS: TRACHEA VS LUNG. DATA FROM MUC5B STAINING OF RESPIRATORY TISSUES**. Tissue sections from each of 8 healthy control horses and 11 RAO-diseased horses from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for RAO and control horses divided into exposed and not-exposed subgroups (according to controlled environmental housing conditions at time of euthanasia) further subdivided into information for separate anatomical areas, trachea (T) and mid-lung bronchi (L) is shown using Kruskall-Wallis ANOVA multiple group comparisons to assess inter-group variation in the parameters A - D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Muc5b dark grey bars. RAO and control (Ctrl) horses were kept in either exposed (E) or not-exposed (NE) controlled environments prior to euthanasia. Graphs show mean +SEM.



For PAS AB staining, the RAO not-exposed T sub-group had significantly larger ($P < 0.001$) mean GC size then their exposed sub-group counterparts (*Fig. 4.22 B*). There was a significantly larger gland staining positive area for both of the RAO T area sub-groups when compared to their L counterparts ($P < 0.01$), however there was no significant difference between any of the sub-groups for the % of gland staining positive for mucin (*Fig. 4.22 C, D*).

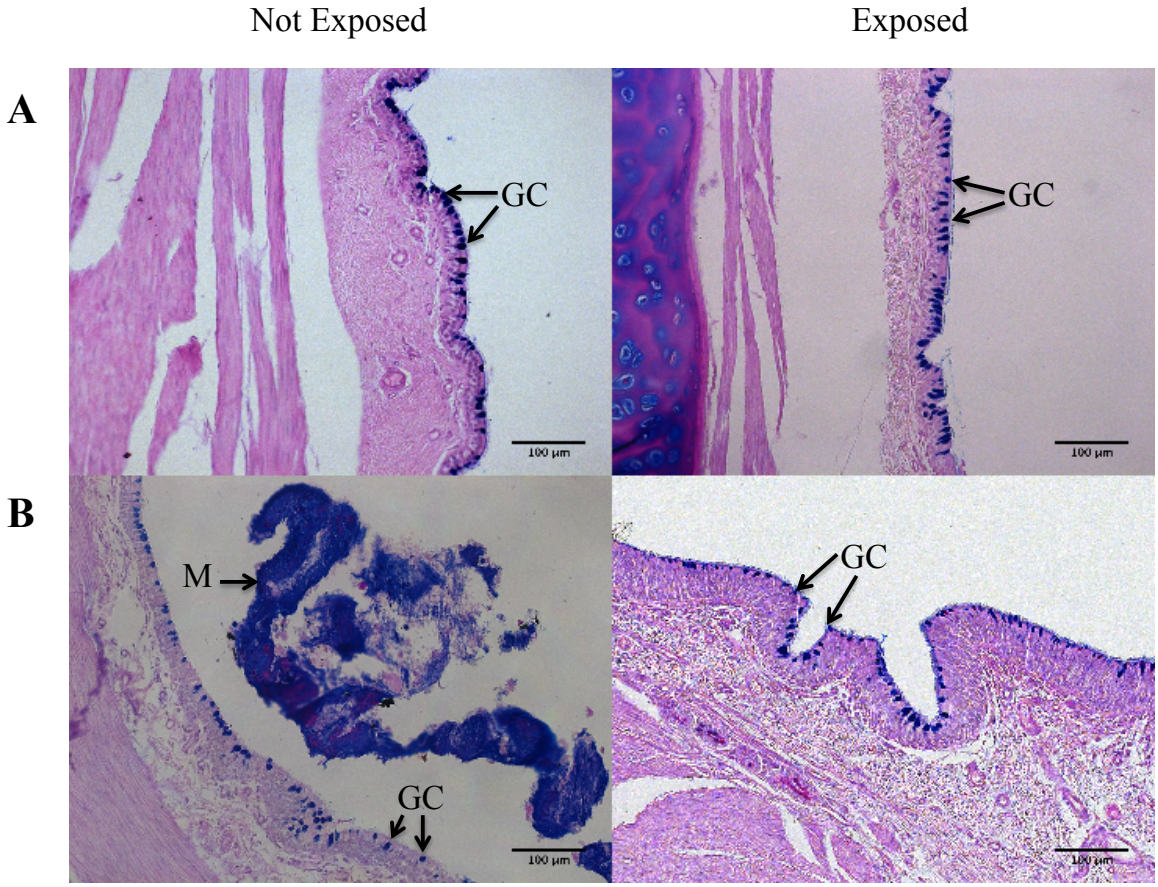
For Muc5b staining, there were significantly more goblet cells per 1000 µm epithelium for the control not-exposed L sub-group compared to the RAO not-exposed L sub-group ($P < 0.01$) (*Fig. 4.23 A*). The control exposed L subgroup had significantly more GC per 1000 µm epithelium than the control exposed T subgroup ($P < 0.05$). Within the RAO horse group subgroups, there were significantly more GC per 1000 µm epithelium in the RAO exposed L subgroup than either of the not-exposed L ($P < 0.0001$) or exposed T subgroups ($P < 0.01$) (*Fig. 4.23 A*). Also, as for PAS AB staining, the control not-exposed L sub-group had significantly larger ($P < 0.0001$) mean GC size then their exposed sub-group counterparts (*Fig. 4.23 B*). There was a significantly larger gland staining positive area for the RAO not-exposed T area sub-group compared to the RAO not-exposed L sub-group ($P < 0.05$); however there was no significant difference between any of the sub-groups for the % of gland staining positive for mucin (*Fig. 4.23 C, D*).

The above information tells us that there are more and occasionally larger GCs for PAS AB stain compared to the Muc5b stain; but no significant differences for the sub-mucosal gland data between the 2 staining methods. We have to bear in mind that this method of Muc5b staining on formalin-fixed tissues is sub-optimal and therefore that could reflect the findings here, or it could be that in some horses, particularly with respect to goblet cells, there is another mucin also contained in the goblet cells, most likely Muc5ac.

Additionally we have established that there are significant differences in epithelial goblet cell numbers between exposed and not-exposed states and in different areas of the respiratory epithelium between and within control and RAO horses. Namely, that control not-exposed horses have more GCs in mid-lung bronchi than not-exposed RAO horses, but that exposed control horses have fewer GCs than exposed RAO horses in both the tracheal and bronchial epithelium. Control exposed horses have significantly more GCs in the bronchial than tracheal epithelium, and this is also the case for exposed RAO horses. Goblet cells are larger in not-exposed than exposed RAO horse tracheal epithelium. Goblet cells are also larger in not-exposed control horse than exposed control horse

bronchial epithelium. The area of gland staining positive for mucin is larger in tracheal than bronchial epithelium for RAO exposed not-exposed horses alike, but the percentage of gland staining positive for mucin is not different between any groups of horses. *Fig 4.24* shows representative PAS AB stained images from each of the 4 sub-groups of horses (control and RAO exposed and not-exposed).

Fig. 4.24 **HEALTHY CONTROL AND RAO HORSE RESPIRATORY EPITHELIUM**. Slides were made from tissue bank samples of respiratory tissue from RAO and healthy control groups of horses, further divided into exposed and not-exposed subgroups, according to controlled environmental housing conditions at time of euthanasia. Slides made from the formalin-fixed paraffin-embedded tissue were stained with general mucin stain PAS AB and counterstained with haematoxylin. Representative images are shown of respiratory epithelium from horses from the not-exposed (left-hand side images), and exposed (right-hand side images) sub-groups of horses from the 2 groups: **A**. Healthy control horses. **B.** RAO diseased horses. Control not-exposed horses were found to have more goblet cells (GC) per unit length of epithelium than RAO not-exposed horses; whilst RAO exposed horses have more GC per unit length of epithelium than control exposed horses. (M) Mucus in airway lumen. Original magnification x 20. Scale bar 100 µm.



4.3 DISCUSSION

Adjustment of the immunohistochemistry technique to enable mucin-specific identification was time-consuming and difficult; however a suitable protocol was achieved at least for identification of Muc5b in ethanol-fixed tissue, comparable to the findings for the general mucin stain, and to an extent, for formalin fixed-tissue also. The comparable Muc5b to PAS AB staining of both epithelial goblet cells and sub-mucosal glands, tells us that many PAS AB positive cells are also Muc5b positive, however it does not tell us if those cells are Muc5ac positive or negative. We did notice some significant differences between Muc5b and the general mucin stain, specifically for goblet cell parameters but not for sub-mucosal gland parameters when examining formalin-fixed tissues from control and RAO horses. We had previously noted that formalin-fixation of tissue was sub-optimal for identification of Muc5b in tissues; however one would expect to concurrently find similar differences in gland data if that was the sole reason for the disparity between PAS AB and Muc5b noted. It has previously been identified that in the human airway at least, MUC5B is produced predominantly in the sub-mucosal glands and MUC5AC more exclusively in the goblet cells. Preliminary data from our laboratory identified that such mucin segregation does not exist in the equine airways. We have to now consider that in some horses examined here, both healthy controls and RAO, there was a difference between general mucin stain and Muc5b in the goblet cells but not the sub-mucosal glands, and therefore Muc5ac or other mucins may account for the difference between the greater GC general mucin stain compared to Muc5b. Perhaps there is a degree of mucin segregation in the equine airways, with Muc5ac being present more commonly in the epithelial goblet cells than the sub-mucosal glands, but as Muc5b is by far the predominant mucin in equine airways, we do not appreciate the segregation as much as the situation in human airways based on analysis of our preliminary data. Alternatively it could be that a subset of horses has more detectable Muc5ac in their airways, whilst some have a strong Muc5b predominance. To further investigate this, work to perfect a Muc5ac-specific antibody for use in immunohistochemistry is needed and so it is unfortunate that we were unable to achieve this during this project. Part of the problem we may have encountered when trying to find a suitable Muc5ac antibody is that if there is little Muc5ac in the equine airways, it may be difficult to get positive identification, therefore future work should critically assess purified Muc5ac antibody fractions used on both ethanol and formalin fixed positive and negative control tissues as well as a range of healthy horse respiratory epithelium.

The healthy horse study samples were taken from horses presented fit for human consumption at an abattoir; therefore there will be large variability in the age, breed, use and management of the individuals, which could in part explain the large inter-horse variability that we observed. We did want to randomly sample from the equine population to get an idea of the extent of variation between horses that may exist. Abattoir horse respiratory tracts were a convenient way of sampling from the healthy horse population; samples which would have otherwise been extremely difficult to source. The collection of abattoir samples was based on whichever animals were presented for slaughter on the day of collection, and therefore the sampling was random in that respect, desirable when wanting to consider the status of mucin-producing glands in the general healthy horse population, but undesirable for effective control of multifactorial variables.

Mucin-producing goblet cells and sub-mucosal glands are present along the length of the equine trachea and primary to tertiary bronchi, contrary to previous findings where sub-mucosal glands have been cited as the major source of mucin in the equine trachea (Widdicombe and Pecson, 2002). There is great variation between healthy horses in the number but not the size of goblet cells, and the size and percentage of mucin producing cells in sub-mucosal glands. There is no difference in goblet cell size or number along the entire length of the respiratory tract (trachea and bronchi) in the healthy horse. The number and size of goblet cells in the epithelium does not vary along the length or around the circumference of the trachea, or between the primary to tertiary bronchi, in healthy horses. This information is contrary to that identified by earlier researchers who identified fewer goblet cells and smaller sub-mucosal glands in the dorsal trachea; the reason for this is unclear; certainly we assessed a larger number of horses and more anatomical areas than previous authors have done. Or, it could be that our larger sample size and repeated measurements at different sections of trachea confounded by a large variation between individual horses, hides any difference. The statistical analysis did take this into account however, by examining the data at 3 different levels (image, slide and horse). The mucin positive gland size is larger in the trachea than bronchi in healthy horses; also the percentage of gland staining positive is larger in the trachea than the bronchi, and bronchial glands have an overall higher percentage staining positive for Muc5b than general mucin stain. This phenomenon is not noted either in the tracheal sub-mucosal glands or in the tracheal or bronchial epithelial goblet cells. Detection of Muc5b can actually be more sensitive than the general mucin stain (Rousseau *et al.*, 2011b), so one would expect the Muc5b stain to consistently produce higher values than for the general mucin stain if all

the mucin detected by the PAS AB stain is Muc5b. The fact that this is only the case in the bronchial sub-mucosal glands and not elsewhere could be due to the bronchial glands producing almost entirely Muc5b, whilst there is a component of other mucins (e.g. Muc5ac) as well as Muc5b (still being the predominant mucin, as shown by our previous biochemical analysis of mucins) in the goblet cells and tracheal sub-mucosal glands. It would be interesting, as pointed out previously, to have parallel Muc5ac data to be able to investigate this further.

Healthy control horses have more Muc5b positive bronchial epithelial goblet cells than RAO horses when horses are housed in an outdoors open-air (not-exposed) environment and the RAO horses are asymptomatic. This is in contrast to the situation when horses are housed in a dusty, (exposed) stable environment where symptomatic RAO horses have more Muc5b positive goblet cells than controls in both the tracheal and bronchial epithelium. Horses housed in an exposed environment have a larger goblet cell count in bronchial epithelium compared to tracheal, for both RAO and control horses. RAO and control horses have smaller goblet cells in tracheal and bronchial epithelium respectively, when kept in an exposed environment. RAO horses have a larger gland size in trachea compared to bronchi, no matter what the exposure status. This is similar to our findings in healthy abattoir horses. We were unable to identify similar findings in the healthy control horses due to a lack of available tracheal tissue from the tissue bank samples, a caveat of using samples collected and processed prior to study design.

4.4 CONCLUSION

Muc5b-5 was the most suitable purified antibody fraction for successful specific identification of Muc5b in mucin cells, along with haematoxylin as a counterstain, and omission of an antigen-retrieval step for ethanol but not formalin-fixed tissues. Muc5b is the predominant mucin in airways mucus and would appear to be the predominant mucin produced in the epithelial goblet cells and mucosal glands in the normal horse, although without Muc5ac data we cannot confirm this.

In healthy and RAO horses, mucins are produced in epithelial goblet cells and sub-mucosal glands throughout the tracheal and bronchial epithelium. Healthy and RAO horses exposed to a dusty challenge environment, have more bronchial than tracheal epithelial goblet cells per length of epithelium than those kept in an open field environment. Symptomatic RAO horses have more goblet cells per length of epithelium than exposed controls in both the bronchi and trachea, whilst the reverse is true in the bronchi of not-

exposed horses. Symptomatic exposed RAO horses have more goblet cells than asymptomatic not-exposed RAO horses, consistent with challenge in a dusty stable environment leading to an increase in number of epithelial goblet cells. This indicates that goblet cell hyperplasia occurs during symptomatic RAO. The size of goblet cells however is larger in not-exposed than exposed horses, in particular in the bronchi of control horses and the trachea of RAO horses. The reason behind smaller goblet cells during periods of exposure-stimulated goblet cell hyperplasia is unclear. It could simply be that the up-regulation of goblet cell numbers in the surface epithelium is at the expense of goblet cell size; or that there is reduced goblet cell apoptosis (Bartner *et al.*, 2006; Lugo *et al.*, 2006). A simple explanation could be that exposure stimulates an increased secretion of mucin, hence their smaller size. Much is yet to be understood regarding mucin secretion at the epithelial surface; however it is widely accepted that there is a baseline level of continual mucin secretion as well as an increased mucin secretion in response to stimuli (Jeffery and Li, 1997; Ordonez *et al.*, 2001). Smaller goblet cell size potentially could reflect reduced mucin synthesis, although that theory is in contradiction to increased endotracheal mucus observed during exposure. Increased endotracheal mucus could also be due to reduced clearance and further investigation of the dynamic situation of mucin production, secretion and clearance is needed to understand this further.

RAO hoses have more glandular mucin in their tracheas than bronchi both during challenge and clinical remission, and although in general we note that the tracheal glands are also larger than the bronchial glands in healthy horses, we cannot unfortunately identify if this is the case during both environmental conditions. The airway epithelium goblet cells and sub-mucosal mucus glands no doubt work synergistically in response to environmental stimuli that leads to the increases in endotracheal mucus noted during exposed conditions. The increased mucin size of sub-mucosal glands of RAO horses could reflect an increased number of mucus cells in the glands or increased storage, identification and counting of cell nuclei could help to resolve this issue. We do not know how the larger size of sub-mucosal gland stored muco-substance affects mucin secretion at the epithelial surface. Studies of gland and epithelial goblet cell morphology during the dynamic process of mucin secretion during exposed and not-exposed conditions will aid further understanding of this process.

We have identified that the increased tracheal mucus in RAO horses during exposure to a challenge environment can be attributed, at least in part, to an increased number of tracheal and bronchial epithelial goblet cells. The sub-mucosal gland size of

RAO horses during exposure is larger than controls, but we do not know if this accounts for some of the increased mucus seen in RAO horse airways compared to healthy horses during exposure. What is less clear is the source of increased tracheal mucus in RAO horses observed when they are asymptomatic, as they appear to have fewer bronchial epithelial goblet cells than their healthy counterparts, and we are unable to fully compare gland size in unexposed groups due to lack of data from not-exposed control horse trachea. The bronchial sub-mucosal gland size in not-exposed horses is not statistically different between RAO and control horses, therefore does the difference lie in the amount of mucin in the tracheal sub-mucosal glands, for which we do not currently have data? Ideally a comparison of tracheal sub-mucosal glands between not-exposed RAO and control horses would help to answer this question. Information regarding the secretion of mucins in RAO and control horses from rigorously controlled environments is also key to understanding why RAO horses also have increased tracheal mucus score while clinically silent. In the following chapter we discuss our attempts to produce a cell-culture system of RAO horse airway epithelium to try to answer this.

CHAPTER 5: AIR-LIQUID INTERFACE CULTURE OF EQUINE AIRWAY EPITHELIAL CELLS

5.1 INTRODUCTION

The preceding chapters have investigated equine airway mucins in mucus samples from horse airways and the cellular distribution of their production sites in the equine airway both in healthy and RAO-diseased horses, to try to establish if there are differences between mucins in health and RAO. We have established that Muc5b is the predominant mucin in both health and in RAO, and that there are increased amounts of mucus, containing the polymeric secreted mucins Muc5b and Muc5ac in RAO horses airways. We have also established that mucins are produced in epithelial goblet cells and sub-mucosal glands in the equine airway, with smaller glands in the smaller airways and a large variation of number of mucin-producing epithelial goblet cells between sample donors. Shortcomings of the preceding experiments have largely been due to the caveats and constraints of sample availability and variability between samples. We initially had access to mucus samples from horses with RAO collected for clinical reasons via tracheal endoscopy, but the problem with this source was largely the lack of healthy control samples for direct comparison, and the large variation between samples in terms of collection method and disease state and environment of the affected animals. We were then fortunate to have access to mucus samples from a controlled experimental herd, in which samples were available from RAO and healthy control animals, which acted as their own controls for exposed and not-exposed environmental conditions. The technique employed to retrieve mucus from the airways of these horses, involving instillation and retrieval of saline trans-endoscopically, yielded inconsistent results: horses had varying amounts of mucus in their airways, it was impossible to reliably quantify and retrieve all mucus present; despite a set volume of saline being injected, equal volumes of mucus were never retrieved. Thus mucus samples collected were in varying dilutions from the outset and contained varying proportions of the mucus present in the trachea. It is also possible, given the viscous, sticky nature of mucus that the thicker mucus from some horse airways was impossible to retrieve via the trans-endoscopic portal due to a limited diameter of the collection tube. Unsurprisingly, the mucin content of the samples collected from these horses, when analysed biochemically, did not correlate with the amount of mucus visualised in the airways, and the technique of sample collection via lavage and suction endoscopically does not necessarily retrieve a representative sample of mucus from the

horse airway. However, we had nevertheless performed these preliminary biochemical investigations on RAO and control horse airway mucus as any alternative methods of mucus collection from the live horse were not available, and euthanasia of diseased horses to permit representative mucus sample collection was not feasible. Whereas in the live horse the problem of mucus sample collection from healthy controls was an issue, when it came to samples of respiratory tissue from euthanased animals, we encountered the opposite problem, that we had access to samples of tissue from freshly killed healthy animals but lacked samples from RAO-diseased animals. Again we were fortunate to obtain samples from a controlled experimental herd of RAO and control horses to perform a histological study of mucin producing cells in the airways, but the samples were historical from a pre-formed tissue bank and so had been processed in a way that was sub-optimum for our techniques. In conclusion, in order to study equine airway mucins in further detail, a rigorously controlled and physiologically robust experimental model is needed.

One of our long-term aims was to make equine airway cell cultures that produce and secrete mucins and mucus to enable us to study them. The previous experiments reported in this thesis have been unable to answer fully what changes occur in mucins in RAO and, importantly, we have not been able to address how their production is regulated and controlled.

Equine airway epithelial cells have been successfully cultured in submerged primary culture, however this culture model does not readily mimic the *in vivo* airway and mucin secretion (Shibeshi *et al.*, 2008; Sime *et al.*, 1997). Researchers have also developed techniques of culturing equine respiratory mucosal explants (Vairo *et al.*, 2013; Vandekerckhove *et al.*, 2009). However the explant models, designed for studying virus interactions in the respiratory mucosa, are maintained on agar and it is difficult to control the basolateral input to the epithelial cells this way and thus it is not a suitable method for the study of mucin production. Cell culture methods designed to mimic *in vivo* respiratory epithelium have been developed giving rise to air-liquid interface (ALI) culture systems (Fulcher *et al.*, 2005). The aim of ALI cultures is to mimic the airway epithelium with ciliated and mucin-producing goblet cells so that mucus can be produced and harvested for analysis under rigorously controlled experimental conditions, where the basolateral influence on the epithelial cells can be tightly controlled. Cell culture methods also negate the use of experimental animals as models, avoiding the associated ethical, welfare, genetic

variability, and financial issues associated with an experimental horse herd. The air-liquid interface system employs a transwell mesh set upon a changeable liquid media layer; epithelial cells are seeded onto the mesh and grown to confluence submerged in media. Once the epithelial layer is confluent, the media on top of the cells is removed and thus the cells are exposed to air apically and the cell culture media basally. The epithelial layer then differentiates (with the addition of retinoic acid in the culture media) forming ciliated cells and mucin-producing goblet cells (Fulcher *et al.*, 2005). The goblet cells produce mucus that is visualised to be transported by the differentiated ciliated cells (Matsui *et al.*, 1998) (Fulcher *et al.*, 2005). Analysis of the mucins produced by human ALI cultures have shown that they are biochemically similar to *in vivo* derived human airway mucins (Thornton *et al.*, 2000).

Air-liquid interface cell cultures have previously been developed for equine airway epithelium in our laboratory and by other researchers (Abraham *et al.*, 2011; Oslund *et al.*, 2010; Quintana *et al.*, 2011; Rousseau *et al.*, 2011a; Schwab *et al.*, 2013; Schwab *et al.*, 2010). The methods that have been thus far employed harvest equine airway epithelial cells from *ex vivo* airways sections, necessitating euthanasia of the donor. Epithelial cells are digested from harvested airway tissue and then grown up on flasks to expand cell numbers and to select purely for epithelial cells (excluding fibroblasts) before transfer to transwell plates for proliferation and differentiation at air-liquid interface. The process of airway collection, enzymatic digestion, cell extraction and plating, and cell expansion takes a minimum of 3-7 days, after which time to full differentiation at ALI takes a minimum of 21 days. In our laboratory at least, cell culture of equine airway epithelium was problematic to develop, with the most commonly encountered problems being fungal contamination of cultures, fibroblast contamination, and failure of cells to adhere and propagate from some donors (K. Rousseau, University of Manchester, personal communication).

The published methods that have been developed for studying equine airways in a controlled experimental model are useful, but they do require euthanasia to enable tissue harvesting. Some of the fundamental problems that we have encountered in this thesis so far have been due to the difficulty of obtaining samples from horses with RAO; the requirement for euthanasia of the animal to enable tissue harvest for cell culture doubly confounds the problem of sample availability as in general, cases of RAO are normally client-owned animals and the condition on its own is not usually reason for euthanasia. Therefore in this chapter we have attempted to address this problem by developing a

sampling technique suitable for use in the live animal to enable study of disease without sacrificing the animal. The cells for ALI cultures from asthmatic human patients are collected by transendoscopic bronchial brushing (Campbell *et al.*, 1993; Kelsen *et al.*, 1992) and so we wanted to investigate if a similar technique would be possible from equine RAO donors. We also examined conditions for freezing harvested cells for later propagation so that if a sample becomes available from a donor and cell culture is for some reason not immediately required, that it can be undertaken at some point in the future.

The aim of this part of this thesis was to develop an ALI model using cells from live donors, to enable the study of mucins from diseased animal cells and investigate how mucin production is regulated and controlled in those cells. The eventual aim of this is to discover therapeutic targets to combat the excessive mucus involved in RAO.

5.2 RESULTS

The technique employed to harvest airway epithelium cells from live human asthmatic donors for ALI culture, that we wish to evaluate for equine epithelial cells, is to harvest cells via transendoscopic brushing (Campbell *et al.*, 1993; Kelsen *et al.*, 1992). Hence we needed to validate that brushing would be a useful and viable methodology for harvesting equine airway epithelial cells to enable ALI culture. In order to investigate this, we performed experiments on freshly harvested equine airways collected from healthy abattoir horses, mimicking the brushing technique to see if it would work prior to performing brushing in the live animal. A number of variations of the protocol were attempted in order to achieve and try to optimize successful ALI culture of equine respiratory epithelium. Cell culture techniques are time-consuming and expensive, so limited numbers of samples and donors were tried at any one time in order to try to identify optimum methodologies.

We have previously identified that the distribution of goblet cells is not different at different portions of the trachea (refer to *Chapter 4*), therefore sampling any section of the trachea should in theory yield cells representative of any portion of the trachea. Sterile dental brushes were used as replica transendoscopic brushes to perform the pseudo-transendoscpic brushing procedure on the *ex vivo* tracheas; the brushes were chosen due to their similar shape and size to transendoscopic bronchial brushes, whilst remaining affordable and readily available. The general procedure for cell collection via brushing is

outlined in the *methods* chapter. In brief the brush was used to collect cells by brushing the epithelium and then transported immersed in transport media on ice before retrieval of the cells from the brush via lavage using the transport media which contained antibacterial and antifungal agents.

5.2.1 DOES TRACHEAL BRUSHING HARVEST ENOUGH CELLS TO CREATE EPITHELIAL CELL PROPAGATION?

Initially, circumferential brushings were taken from tracheal sections; we wanted to establish if an increased number of circumferential brushings provided a better yield of cells, i.e. if there were a minimum number of times a brush needed to be passed around the circumference of the trachea to yield a sufficient number of cells that would proliferate in submerged culture suitable for transfer to ALI. In the live animal, an increased number of circumference brushings may lead to petechial haemorrhages and localized inflammation and may be less well tolerated, so our aim was to gauge a balance between minimal tissue damage and sufficient cell collection.

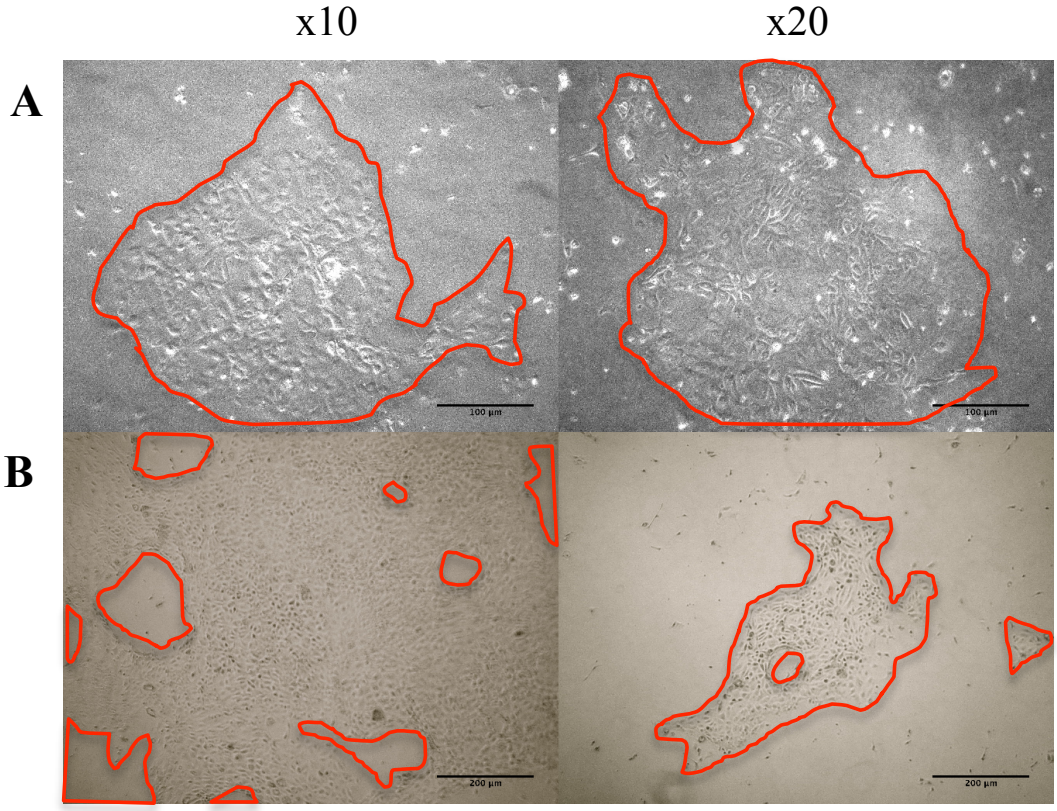
We initially chose the parameters of 1, 5, 10 and 20 times around the circumference of the trachea to assess this question, and performed these 4 brushing regimes in the tracheas of 2 donor abattoir horse tracheas (H1 and H2). The collected cells were seeded onto vented, collagen-coated T75 flasks. All 4 samples from H2 had fungal infection and had to be discarded. For H1, there was no cellular growth for the x1 circumferential brushing while the x5 brushing developed a fungal infection by day 4. The x10 and x20 brushings had established typical cobblestone epithelial cellular growths by day 4 post-collection, which had expanded by 7 days post-collection, > 50 % for the x10 brushing flask and about 15 % of surface covered for the x20 flask (*Table 5.1, Fig. 5.1*).

From these data we concluded that one circumferential brushing was not sufficient, and as we did not have any data on 5x brushing, we decided to continue by looking at x10 and x20 circumferential brushings, which had, at least from one donor, led to propagation suitable for transfer to ALI transwell inserts based on previous published information (Oslund *et al.*, 2010; Schwab *et al.*, 2010).

Table 5.1 **TESTING CIRCUMFERENTIAL TRACHEAL BRUSHING TO CULTURE EQUINE RESPIRATORY EPITHELIAL CELLS: DONORS H1 AND H2.** Equine respiratory epithelial cells were harvested from tracheas of 2 healthy equine donors, (H1 and H2) immediately post mortem using a separate sterile brush for each brush collection. Brushings were performed around the circumference of a 3 cm section of the tracheal epithelium 1, 5, 10 or 20 times (x1, x5, x10, x20 respectively) to enable cell harvest. Brushes were transported (on ice) in transport medium containing antibacterial and anti-fungal agents. Cells were collected via gentle lavage of the brush head and pelleting by centrifugation (1000 rpm, 3 mins) and then seeded onto collagen-coated vented T75 culture flasks submerged in equine airway culture medium and 10 % FBS at 37 ^{o}C in a 5 % CO_2 environment. The table identifies final outcome of culture of each cell brushing from each donor. (-) cells did not adhere or proliferate; (F) fungal infection; (+) some epithelial cell growth limited to < 15 % confluence; (++) epithelial cell proliferation to around 50 % confluence.

	Circumferential brushings			
Donor	**x1**	**x5**	**x10**	**x20**
H1	-	F	++	+
H2	F	F	F	F

Fig. 5.1 **PHOTOMICROGRAPHS OF CELL CULTURE CELLS FROM DONOR H1**. Cells were harvested via brushing the tracheal epithelium immediately post mortem and seeded onto collagen-coated T75 culture flasks and incubated in 5 % CO_2 at 37 ^{o}C in equine airway culture medium (see *Methods*). x10 (left hand images) or x20 (right hand images) circumferential brushings were taken. Cobblestone appearance of epithelial cells adherent to culture flask is shown at **A**. day 4 after seeding, 40 x original magnification. **B**. day 7 after seeding, 20 x Original magnification. Red lines approximately delineate edges of epithelial cell clusters.



5.2.2 CAN LONGITUDINAL BRUSHING OF THE TRACHEA ALSO HARVEST ENOUGH CELLS FOR EPITHELIAL CELL PROPAGATION, AS IS THE CASE WITH CIRCUMFERENTIAL BRUSHING? CAN THESE CELLS BE STORED VIABLY AT -80 ^{O}C FOR LATER PROPAGATION?

On consideration of the practicalities of brushing the epithelium *in vivo*, we contemplated that circumferential brushing may be technically more difficult to perform via the endoscope, and so we wanted to investigate if longitudinal brushing of the trachea would yield sufficient cells for culture. We were also interested to investigate if seeding cells from brushing directly onto transwell plates rather than propagating first in flasks was

an alternative method to achieve ALI culture from brushings. Another question for consideration was if cells collected via brushing can be stored frozen following collection and later proliferated and differentiated in ALI?

In order to investigate the 3 questions outlined above, tracheas were collected from 3 donor horses (H3 - H5) immediately after slaughter. Cell brushings were collected in triplicate from each trachea via circumferential brushing (x10 and x20) and also longitudinal brushing. The longitudinal brushing performed was 1x or 4x up and down the mid section of the trachea, to mimic what may be feasible trans-endoscopically. Cells from each brushing method were plated separately directly onto flasks and transwell plates; the third set of cell brushings was pooled for each horse (both longitudinal and circumferential methods combined) and was frozen at -80 ^{o}C in equine airway culture medium containing 10 % FBS and 10 % DMSO.

5.2.2.1 SEEDING CELLS DIRECTLY ONTO TRANSWELL INSERTS

Firstly, the cells that were seeded directly onto transwells were not successful for any brushing method for any horse. Each of the 2 longitudinal brushings for both H3 and H4 all had fungal infections. Cells from the circumferential brushings from H4 did not adhere or proliferate. Small areas of epithelial cell cobblestone growth occurred for all brushings (longitudinal and circumferential) from H5, and for the circumferential brushings from H3; however growth was over small areas of the transwell plates and never exceeded 10 - 15 % of the transwells, hence ALI culture was not feasible in these cultures (*Table 5.2*).

5.2.2.2 SEEDING FRESHLY HARVESTED CELLS ONTO CULTURE FLASKS

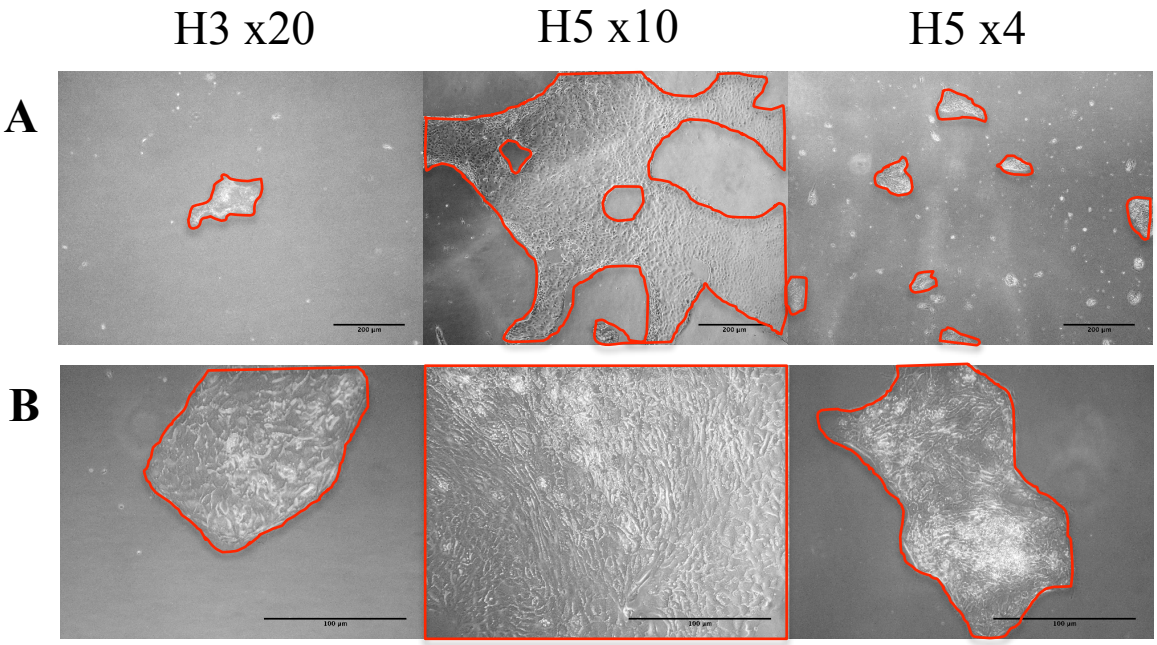
Of the cells that were seeded from brushing onto collagen-coated flasks, all brushings from H4, and the x4 longitudinal brushing from H3 developed fungal infections and were discarded. There was no cell adherence and growth from the x1 longitudinal brushing from H3. Both the x10 and x20 circumferential brushings from H5 grew epithelial cells with a cobblestone appearance submerged in media with 80 – 90 % confluence on the flasks by day 18 post seeding (*Fig. 5.2*). Small areas of cobblestone epithelial growth were present in the flasks of H3 for x10 and x20 circumferential brushings, and H5 x1 and x4 longitudinal brushings. The cells in these flasks were treated with trypsin-EDTA to separate the cells and then the trypsin neutralized with trypsin neutralising solution and the cells left in the same flasks to encourage adherence and colonization of a larger flask area.

This did not lead to a confluent growth by 18 days post collection and cells were subsequently discarded (*Table 5.2*).

Table 5.2 **TESTING CIRCUMFERENTIAL AND LONGITUDINAL TRACHEAL BRUSHING TO CULTURE EPITHELIAL CELLS: DONORS H3, H4 AND H5.** Equine respiratory epithelium cells were harvested from tracheas of 3 healthy equine donors (H3, H4 and H5), immediately post mortem using sterile brushes. Brushings were performed in triplicate from each donor, around the circumference of a 3 cm section of the tracheal epithelium 10 (x10) or 20 (x20) times, or up and down the length of the central tracheal epithelium 1 (x1) or 4 (x4) times to enable cell harvest. Brushes were transported (on ice) in transport medium containing antibacterial and anti-fungal agents. Cells were collected via gentle lavage of the brush head and pelleting by centrifugation (1000 rpm, 3 mins) and then seeded either immediately or after storage at -80 ^{o}C (-80), on collagen-coated vented T75 culture flasks; or seeded onto ALI transwell inserts (TrW). Seeded cells were cultured submerged in equine airway culture medium and 10 % FBS at 37 ^{o}C in a 5 % CO_2 environment. Cell brushings collected for frozen storage were pooled for each donor and frozen in equine airway culture medium containing 10 % FBS and 10 % DMSO. The table identifies final outcome of culture of each batch of cells from each donor. (-) cells did not adhere or proliferate; (F) fungal infection; (+) some epithelial cell growth limited to < 15 % confluence; (++) epithelial cell proliferation to around 50 % confluence; (+++) epithelial cell proliferation to > 90 % confluence; (*) trypsin *in situ* cell separation performed; (/) culture not performed in this sample.

	Longitudinal brushing				**Circumferential brushing**				**mixed**
	x1		x4		x10		x20		
Donor	**TrW**	**T75**	**TrW**	**T75**	**TrW**	**T75**	**TrW**	**T75**	**-80**
H3	F	-	F	F	+	+*	+	+*	/
H4	F	F	F	F	-	F	-	F	/
H5	+	+*	+	+*	+	+++	+	+++	-

Fig. 5.2 **REPRESENTATIVE PHOTOMICROGRAPHS OF CELL CULTURE CELLS FROM CIRCUMFERENTIAL AND LONGITUDINAL TRACHEAL BRUSHINGS FROM DONORS H3 AND H5**. Cells were harvested via brushing the tracheal epithelium immediately post mortem and seeded onto collagen-coated T75 vented culture flasks and incubated in 5 % CO_2 at 37 ^{o}C. Images are shown for donors H3 and H5 at **A**. day 4 post seeding (10 x original magnification). **B**. 18 days post seeding (40 x original magnification) onto flasks. Red lines approximately delineate edges of epithelial cell clusters, red rectangle indicates > 90 % confluence in H5 x10 d18.



From these data, we cannot conclude that longitudinal brushing as well as x10 - x20 circumferential brushing could be used as a method for collecting cells that would proliferate in submerged culture. We continued to use both brushing methods in further experiments to try to ascertain whether the longitudinal method could be useful.

5.2.2.3 FROZEN STORAGE OF CELLS PRIOR TO SEEDING ONTO T75 CULTURE FLASKS: DO CELLS PROPAGATE?

Given that donor H5 had yielded the most successful cultures from the fresh brushing to culture flasks, we assumed that cells from this horse may have the most favorable chance to propagate after being stored frozen after brushing collection. A vial of frozen H5 cells was defrosted and seeded onto 2 culture flasks. Live cells were present and adhered to the collagen-coated flasks but after one month had not propagated and so were discarded.

We were investigating if it was possible to freeze cells collected at brushing and later use them for ALI culture with the aim of collecting epithelial cells from RAO-diseased horses and using them in future experiments so that diseased horse cell-lines could be studied. In the preceding experiments, cells collected via brushing and freshly seeded onto culture flasks led to an epithelial monolayer useful for ALI culture; whilst neither seeding directly onto transwell inserts or freezing prior to propagation managed to achieve this. These results were on the basis of cells grown from one donor (H5), so it was considered worth retrying the freezing process with brushings from more donors. Circumferential (x10, x20) and longitudinal (x1, x4) brushings were taken from 3 new donors, H6 - H8, cells were collected from brushes following the same protocol and then frozen at -80 ^{o}C in 10 % FBS 10 % DMSO in equine cell culture media. The concentration of antifungal agent in the cell transport and culture media was doubled in an attempt to control fungal infections.

After one week at -80 ^{o}C, cells were defrosted and seeded onto collagen-coated culture flasks. All flasks from donor H8, and x1 longitudinal brushing flask from H7 had fungal growth and were discarded. The other flasks had a large number of visibly viable cells but these cells failed to adhere and propagate; 10 % serum was added to the equine airway culture medium to encourage growth but the cells did not grow and after 21 days were discarded (*Table 5.3*). The increased concentration of anti-fungal agent in the media had not prevented fungal growth in the cultures, so in future experiments the double concentration was not used as it had not had the desired effect and could potentially interfere with epithelial cell growth.

Table 5.3 **TESTING CIRCUMFERENTIAL AND LONGITUDINAL TRACHEAL BRUSHING WITH FROZEN STORAGE PRIOR TO CULTURE OF EPITHELIAL CELLS: DONORS H6, H7 AND H8**. Equine respiratory epithelium cells were harvested from tracheas of 3 healthy equine donors (H6, H7 and H8), immediately post mortem using sterile brushes. Brushings were performed around the circumference of a 3 cm section of the tracheal epithelium 10 (x10) or 20 (x20) times, or up and down the length of the tracheal epithelium 1 (x1) or 4 (x4) times, to enable cell harvest. Brushes were transported (on ice) in transport medium containing antibacterial and anti-fungal agents. Cells were collected by gentle lavage of the brush head and pelleting by centrifugation (1000 rpm, 3 mins) and then frozen in equine airway culture medium containing 10 % FBS, 10 % DMSO and double the normal culture medium concentration of antifungal agent, at -80 ^{o}C, prior to seeding on collagen-coated vented T75 culture flasks. Seeded cells were cultured submerged in equine airway culture medium and 10 % FBS at 37 ^{o}C in a 5 % CO_2 environment. The table identifies final outcome of culture of each batch of cells from each donor. (-) cells did not adhere or proliferate; (F) fungal infection.

	Longitudinal brushing		**Circumferential brushing**	
Donor	**x1**	**x4**	**x10**	**x20**
H6	-	-	-	-
H7	F	-	-	-
H8	F	F	F	F

5.2.2.4 RECOVERING CELLS COLLECTED VIA BRUSHING AND THEN STORED AT -80 ^{O}C UP TO ALI

The current method was producing cells from brushing that could survive frozen storage but not adhere and proliferate on flasks in submerged culture following freezing, and so reassessment of the method was required. It is possible that some cells from some individual donors simply do not propagate as well as cells from others, so plausibly the previous three donors (H6 - 8) had cells that would not propagate well, which could be one

reason accounting for the lack of growth in those samples. We decided to increase the amount of serum (FBS) in the media when the defrosted cells were seeded (from 10 % to 20 %) to determine if that had a positive effect on cell propagation. Cells were collected via brushing from 3 further donors (H9 - H11) and frozen as for the previous 3 donors. Two vials of frozen cells from each donor (x10 circumferential and x4 longitudinal) were defrosted and plated onto separate flasks with equine cell culture media containing 20 % FBS. One flask from donor H11 developed a fungal infection and was discarded, the other failed to produce any areas of significant cell growth and after one month was discarded. The x4 flasks for donors H9 and H10 grew multiple small cobblestone appearance epithelial cell growth but then after 4 days developed fungal infections and had to be discarded. The x10 flasks from both donors H9 and H10 had multiple areas of epithelial cell growth and expansion to typical cobblestone appearance by 2 weeks after seeding (*Fig. 5.3; Table 5.4*). In situ trypsin treatment of the cells in these flasks was performed with trypsin neutralization to allow the cells to spread out across the flask and encourage growth to confluence. For donor H9 this led to cell growth and expansion to cover about 50 % of the flask surface, but no more growth was achieved after 3 weeks. For donor H10, in situ trypsin treatment led to rapid expansion of the epithelial cells and seven days later, over 95 % confluence with typical epithelial cobblestone appearance was achieved (*Fig. 5.4*). The cells were separated from the flask using trypsin and seeded onto transwell inserts. After 5 days on transwell inserts, confluence was achieved and the apical media layer was removed, creating ALI culture (*Fig. 5.4*). Retinoic acid was added to the media to encourage differentiation.

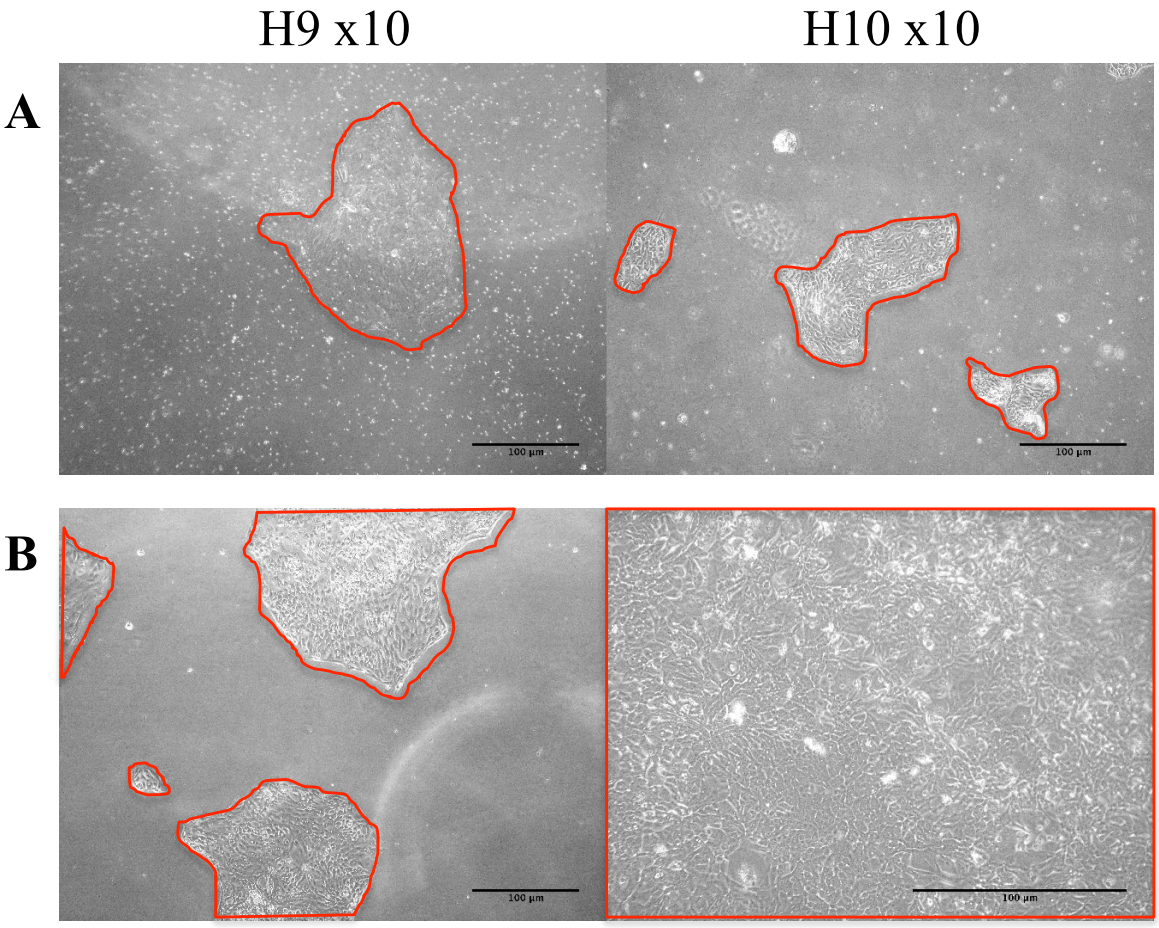
The ALI cultures remained viable for 20 days before cellular deterioration occurred. Cell washing and conditioned (basal) media (that the cells were growing on top of) were collected from the ALI cultures at weekly intervals and probed for mucin presence using slot blot and Western blotting, PAS staining and immunodetection for the major respiratory mucins Muc5b and Muc5ac. Mucins were identified in the cell washes by positive PAS, Muc5b and Muc5ac staining on the slot blot (*Fig. 5.5*). Mucins were also detected in the basal conditioned media. This suggested that the cell layer was leaky because mucins secreted on the apical surface were detected in the basal media. The cell washes and conditioned media were used to perform agarose gel electrophoresis (in duplicate) with probing for equine Muc5b and Muc5ac; however no mucins were detected

on the gel analysis, most likely due to the low levels of mucin present in the washes (data not shown).

Table 5.4 **TESTING CIRCUMFERENTIAL AND LONGITUDINAL TRACHEAL BRUSHING WITH FROZEN STORAGE PRIOR TO CULTURE OF EPITHELIAL CELLS: DONORS H9, H10 AND H11**. Equine respiratory epithelium cells were harvested from tracheas of 3 healthy equine donors (H9, H10 and H11), immediately post mortem using sterile brushes. Brushings were performed around the circumference of a 3 cm section of the tracheal epithelium 10 times (x10), or up and down the length of the tracheal epithelium 4 times (x4) to enable cell harvest. Brushes were transported (on ice) in transport medium containing antibacterial and anti-fungal agents. Cells were collected via gentle lavage of the brush head and pelleting by centrifugation (1000 rpm, 3 mins) and then frozen in equine airway culture medium containing 10 % FBS, 10 % DMSO at -80 ^{o}C, prior to seeding on collagen-coated vented T75 culture flasks. Seeded cells were cultured submerged in equine airway culture medium and 20 % FBS at 37 ^{o}C in a 5 % CO_2 environment. Table identifies final outcome of culture of each batch of cells from each donor. (-) cells did not adhere or proliferate; (F) fungal infection; (++) epithelial cell proliferation to around 50 % confluence; (+++) epithelial cell proliferation to > 90 % confluence; (*) following trypsin in situ cell separation; (ALI) cells taken successfully to air-liquid interface culture.

	Circumferential brushing	**Longitudinal brushing**
Donor	**x10**	**x4**
H9	++*	++ F
H10	+++ * ALI	++F
H11	F	-

Fig. 5.3 **REPRESENTATIVE PHOTOMICROGRAPHS OF CELL CULTURE CELLS FROM DONORS H9 AND H10**. Cells were harvested via brushing epithelium of trachea immediately post mortem and frozen at -80 ^{o}C for 1 week before defrosting and seeding onto collagen-coated T75 vented culture flasks and incubated in 5 % CO_2 at 37 ^{o}C. Images are shown for donors H9 and H10 at **A**. day 14 (20 x original magnification) and **B**. day 21 (20 x original magnification for H9, 40 x original magnification for H10) post seeding onto flasks. Red lines approximately delineate edges of epithelial cell clusters, red rectangle indicates > 90 % confluence.



We had now managed to achieve ALI culture of equine airway epithelial cells from one donor horse, collected via brushing and frozen before propagation, seeding the defrosted cells in equine airway cell culture media supplemented with 20 % FBS. The process of growth and expansion of frozen cells to achieve ALI cultures had taken over a month and required trypsin treatment of cells in situ to spread the cells over the flask and encourage propagation over a wider area of the flasks.

Fig. 5.4 **REPRESENTATIVE PHOTOMICROGRAPHS OF ALI CELL CULTURE CELLS FROM H10**. Cells were harvested via brushing the tracheal epithelium immediately post mortem and frozen at -80 ^{o}C for 1 week before defrosting and seeding onto collagen-coated T75 vented culture flasks and incubated in 5 % CO_2 at 37 ^{o}C and then seeded onto transwell inserts after 3 weeks' propagation. **A**. submerged culture on transwell inserts at day 1 (left image, 40 x original magnification) and day 2 (right image, 20 x original magnification) **B**. ALI culture at day 7 (left image) and day 14 (right image) of ALI culture (10 x original magnification). Red lines approximately delineate edges of epithelial cell clusters. Red arrows point to single epithelial cells adherent to culture flask, red rectangle indicates > 90 % confluence.

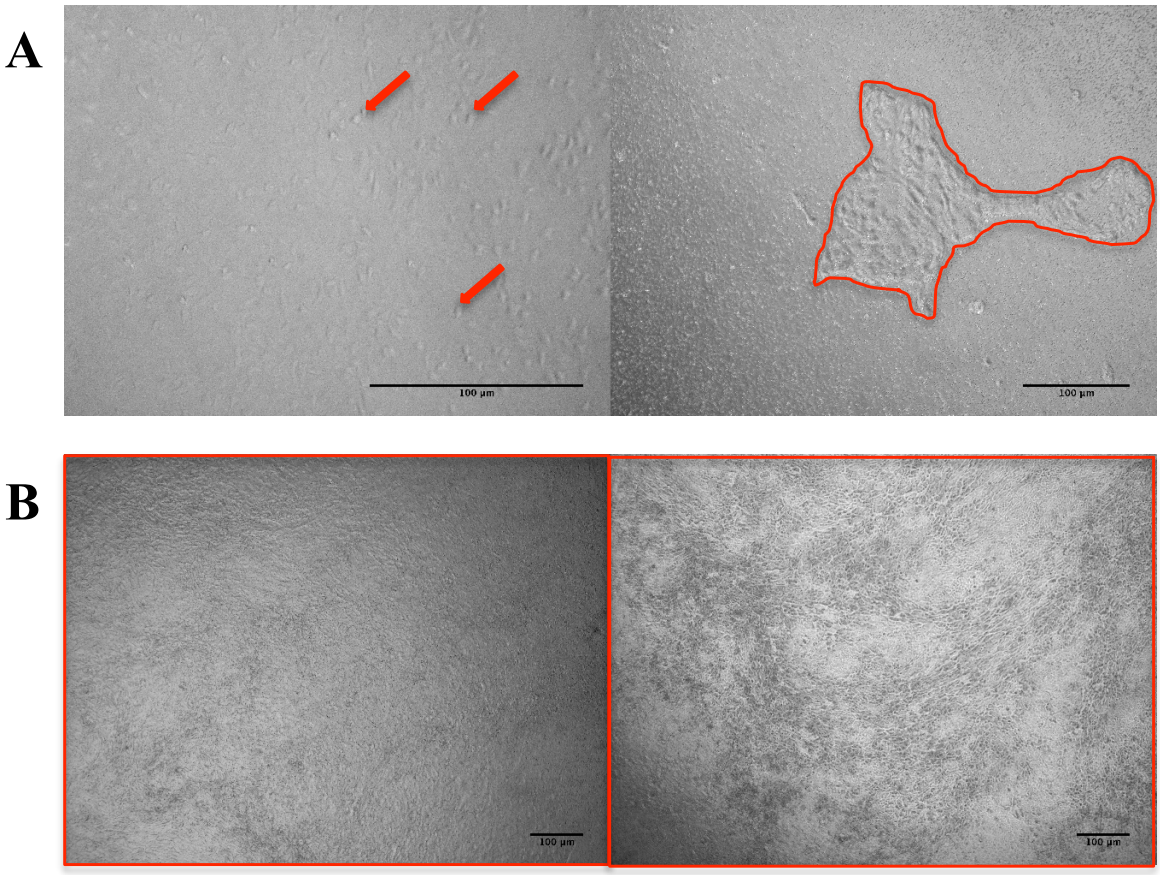
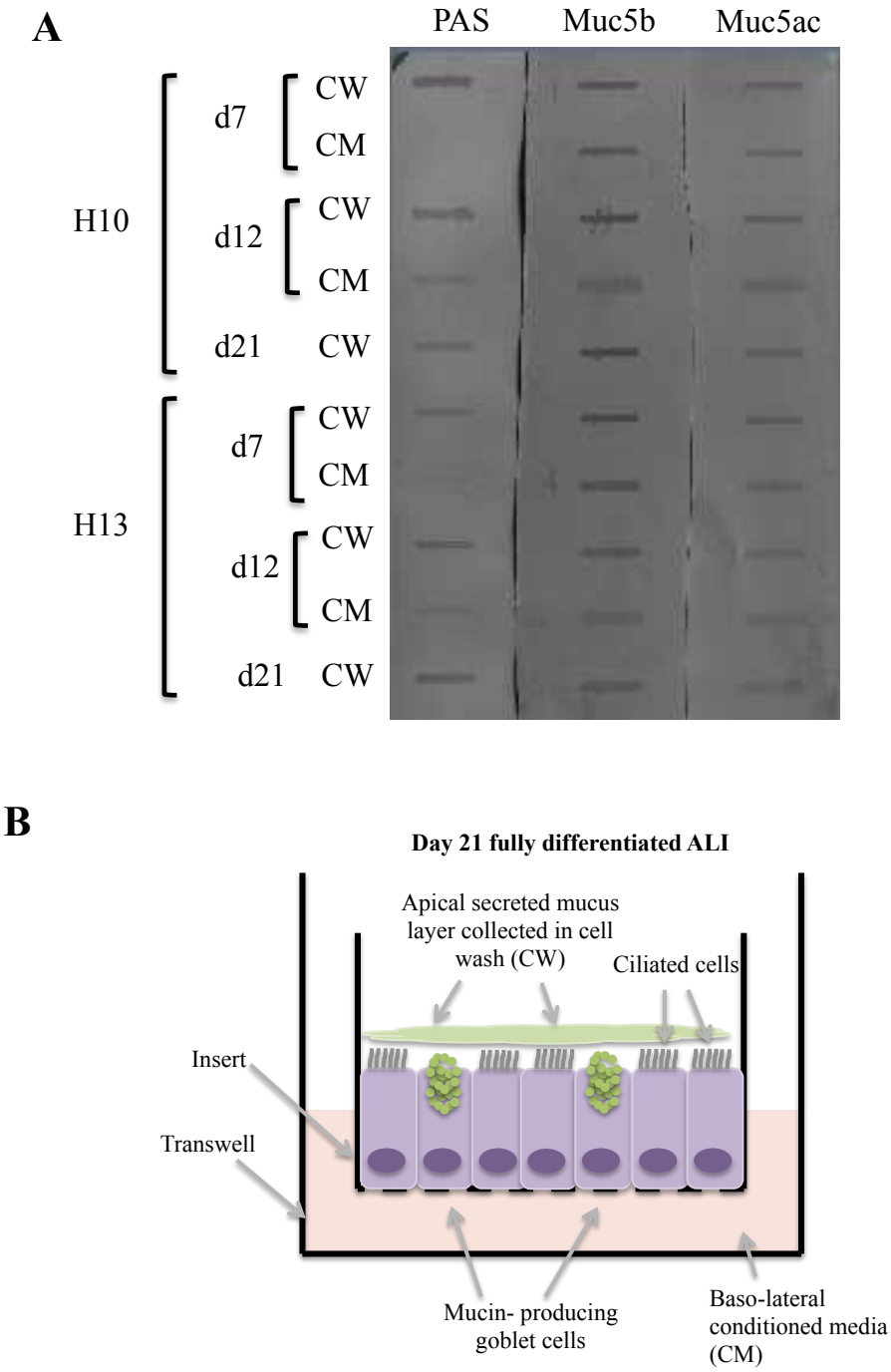


Fig. 5.5 **MUCIN DETECTION IN CELL WASHES (CW) AND CONDITIONED MEDIA (CM) FROM ALI CULTURES FROM DONOR HEALTHY HORSES H10 AND H13**. **A.** Slot blot and mucin detection using PAS, and antibodies for equine Muc5b and Muc5ac. PAS 100 µL/ well, immunodetection 50 µL/ well. CW = apical cell washing; CM = basal conditioned media B. Schematic drawing to represent ALI culture model, not to scale.



5.2.2.5 TRACHEAL DIGESTION AND TRACHEAL BRUSHING: COMPARISONS FROM THE SAME DONORS

Previous attempts at equine airway epithelium ALI culture in our laboratory revealed a variation between donors in ability of cells to adhere and proliferate. Therefore we wanted to make sure that when testing the brushing method that cells from the donor horses would at least grow and proliferate via the published ALI method, so that if the brushing technique failed we could know that this was not simply because donors were unsuitable. To do this, tracheal sections collected from 4 healthy abattoir horses (H12 – H15) immediately following slaughter were cut into two equal length halves (cranial and caudal) to employ the published method on one half, and to practice the brushing technique (circumferential x10) on the other half. The cranial and caudal halves were randomly assigned to either method because our histological study has shown that there are no differences in epithelial goblet cell distribution along the length of the trachea.

The published method of tracheal digestion was undertaken and cells were seeded onto 4 culture flasks per horse; we chose to seed onto 4 flasks from the start of this experiment to avoid complete loss of cells from a donor if one flask succumbed to fungal infection. All four flasks from one donor (H14) succumbed to bacterial infection and were discarded (*Table 5.5*). Two flasks from one donor (H15) were discarded due to fungal infection, whilst the remaining 2 flasks from this donor did have some epithelial growth with cobblestone appearance; however proliferation was slow and only 5 – 15 % of the flask became covered with epithelial cells and seeding to transwell failed to produce confluence to support ALI culture. One donor (H12) had one flask that became infected with fungus within 2 days of seeding, while another flask from this donor quickly had approximately 85 % confluence with epithelial cobblestones before also becoming infected with fungus (*Table 5.5*). The remaining 2 flasks from this donor grew 15 % and 50 % confluence with epithelial cells before a distinct fibroblast population rapidly proliferated and grew concurrently. Attempts were made to separate the two cell populations by trypsin treatment of cells and removal of media from the flask and trypsin neutralization at different time points with seeding onto separate flasks. This was not successful; the flask on which the epithelial population was seeded later became overgrown with cells of a fibroblast phenotype. The final donor (H13) had 2 flasks that had to be discarded due to fungal infection, one flask that became 50 % confluent with epithelial cobblestone

appearance cells before a bacterial infection destroyed the cells, and one flask that grew an epithelial cell population to over 90 % confluence, which was then seeded onto transwell inserts and successfully taken to ALI (*Table 5.5*). Cell washes and cell media was collected from the ALI cultures and used for slot blot and agarose gel electrophoresis mucin detection as for the ALI cultures from donor H10 (*Fig. 5.5*). The slot bot detection revealed mucin presence in the cell surface washings aswell as the basal conditioned media, as had been the case with the previous ALI cultures; and again no mucins were detected on agarose gel blots (data not shown).

The technical difficulties even in using the published method to take equine airway epithelial cells to ALI can be appreciated by examining the above experiment, where only one flask out of 16 was successfully taken to the ALI stage.

Cells collected via brushing from the above donors (H12 - H15) were used for seeding cells onto flasks for immediate cell propagation following collection, and cells were also frozen for later propagation. The freshly seeded brushing cells from donor H12 rapidly expanded with epithelial cells of a cobblestone appearance, but then the cell growth was overtaken with cells of a fibroblast phenotype. The brushing cells from donor H12 that were frozen prior to seeding had some epithelial cell growth within one day of seeding but the flask then acquired fungal infection and were discarded. Brushing cells from donor H13, which had been successfully taken to ALI with the published method of tracheal digestion, grew to 50 % confluence when freshly seeded, but never achieved more growth suitable for seeding to transwells; and although initially epithelial cells grew following freezing, these cells then developed a fungal infection and were destroyed. Both freshly seeded and frozen prior to later seeding flasks for donor H14 had fungal infection and were discarded. No significant epithelial growth occurred in any of the flasks of brushing collected cells from donor H15 (*Table 5.5*).

Table 5.5 **TESTING PUBLISHED METHOD OF TRACHEAL DIGESTION HARVEST OF CELLS, AND TRACHEAL BRUSHING METHODS PLATED DIRECTLY ONTO CULTURE FLASKS IMMEDIATELY OR FOLLOWING FROZEN STORAGE, TO CULTURE EPITHELIAL CELLS FROM THE SAME HORSE: DONORS H12, H13, H14 AND H15.** Equine respiratory epithelium cells were harvested from tracheas of 4 healthy equine donors (H12, H13, H14 and H15) immediately post mortem. Each trachea was divided into two halves and one half used to harvest epithelial cells by the published method of tracheal digestions (Schwab *et. al*., 2010). The other half of each trachea was used to harvest cells via longitudinal brushing of the epithelium, using a sterile brushes. Tracheal sections and brushes were transported (on ice) in transport medium containing antibacterial and anti-fungal agents. Cells were collected from brushes via gentle lavage of the brush head and pelleting by centrifugation (1000 rpm, 3 mins) and then seeded either immediately or after storage at -80 ^{o}C (-80) on collagen-coated vented T75 culture flasks. Cells collected via the published tracheal digestion method were seeded onto 4 collagen-coated vented T75 culture flasks and cultured submerged in equine airway culture medium and 10 % FBS or 20 % FBS (if frozen first) at 37 ^{o}C in a 5 % CO_2 environment. The table identifies final outcomes of culture of each batch of cells from each donor. (-) cells did not adhere or proliferate; (F) fungal infection; (B) bacterial infection; ($) fibroblast proliferation; (+) some epithelial cell growth limited to < 15 % confluence; (++) epithelial cell proliferation to around 50 % confluence; (+++) epithelial cell proliferation to > 90 % confluence; (^) trypsin treatment and separation of epithelial and fibroblast cell populations attempted; (ALI) cells taken to air liquid interface culture.

Donor	**T75**	**-80**	**Tracheal digestion**			
H12	++ $	+F	F	+++F	+ $^	++ $^
H13	++	+ F	F	F	++B	+++ ALI
H14	F	F	B	B	B	B
H15	-	-	F	F	+	+

5.3 DISCUSSION

In this chapter our aims had been to establish a method of harvesting cells from live donors so that in the future cells from RAO horses could be cultured and their mucin production studied. Previously published methods of equine ALI culture utilised cells harvested post mortem, making sample availability from RAO horses limited and unreliable; the aim of developing a method to harvest cells from live donors was to bypass the requirement for euthanasia. Asthma is a human disease with many similarities to RAO, and so we chose to investigate use of a method used in asthmatic patients of harvesting airway cells from live donors by transendoscopic brushing that could be readily performed in live horses (Campbell *et al.*, 1993; Kelsen *et al.*, 1992; Romagnoli *et al.*, 1999). We used tracheas from healthy horses immediately post euthanasia, to assess if use of a brush to collect tracheal epithelial cells could work to bring equine airway cells up to ALI, before testing the technique in live animals.

Our initial investigations established that brushing around the circumference of the trachea between 10 to 20 times collected an adequate cell number for growth and proliferation of equine airway cells on culture flasks, which would be suitable for transfer to ALI culture. Brushing fewer times than this may yield inadequate cell numbers for successful propagation and eventual ALI culture. We also established that seeding of brushing cells directly onto ALI transwell plates was not a suitable technique to achieve ALI, and cells from brushing require propagation in culture flasks prior to transfer to ALI. Results of analysis of brushing longitudinally in the trachea were inconclusive; some successful growth of cells in culture flasks was achieved but confirmation of suitability for transfer to ALI was thwarted by problems of fungal contamination ruining cultures.

We were successful in establishing that cells collected by brushing that are frozen in 20 % FBS can be thawed and proliferated to suitable numbers sufficient to be taken to ALI. We also found that in situ trypsin treatment of small clusters of epithelial cobblestones that are slow growing on culture flasks can be successful in producing cellular proliferation to confluence for cells to be later taken to ALI.

The mucin detection in cell washings and basal media collected from the few successful ALI cultures reported here demonstrate that in those cultures at least, the cellular layer was permeable, i.e. that there were holes in the epithelial cell layer cell junctions. This is far from ideal for an ALI model, as it does not mimic the *in vivo*

situation. If better results can be achieved from cell brushing leading to cell propagation and ALI in the future, then this is something that will need to be addressed.

The theory of this experimental model is excellent in principle, but the practicalities are rather more frustrating. ALI culture of equine airway cells is a difficult and time-consuming technique, whether cells are harvested by the published method or otherwise. The difficulty we had in this study in establishing ALI culture using the published method of tracheal digestion goes to highlight problems encountered with the technique. The most commonly encountered problem was fungal contamination of cultures; despite rigorous adherence to a sterile technique and regular cleaning and disinfection of incubator, hoods and apparatus. Horses are often housed in environments rich with fungal spores (in hay feed and straw bedding), and this may be the reason behind the high levels of fungal contamination noted in these reported attempts at cell culture. To further confound this, fungal spores are frequently reported in tracheal lavage and broncho-alveolar lavage washings from RAO horses, more so than in non-RAO airway washing samples (Larson and Busch, 1985); thus culture of cells from RAO horse airways may never be easily achievable.

There appears to be a huge variation in growth and proliferation of cells between donor horses; we were unable to investigate the reason for this within the limited scope of this study, but horse age, breed and housing environment could be important determinants of this. Apart from fungal growth, and inter-horse donor variations in cell propagation, we also encountered problems of bacterial contamination, and overgrowth of epithelial cells with cells of a fibroblast phenotype.

Positive findings that can be taken away from this preliminary and exploratory study are that, under some circumstances, use of a brush to collect equine airway epithelial cells can lead to successful cellular propagation to confluence suitable for use in ALI. We have also found suitable conditions in which cells collected via brushing can be frozen and successfully grown at a later date. However, for the use of trans-endoscopic brushing for collection of live horse airway cells for ALI culture to become a viable method, the methodology requires improvement to make repeatable reliable results achievable.

5.4 CONCLUSIONS

Air liquid interface culture of equine airway cells is a difficult and time-consuming technique, thwarted by problems of fungal, bacterial and fibroblast overgrowth of cultures,

and hindered by difficulties in obtaining samples from live donors and differences in ability of cells to grow between donors. Circumferential brushing of the equine tracheal epithelium can feasibly harvest sufficient cells suitable for growth and propagation before or after freezing, with eventual seeding and differentiation to ALI. Refinement of methods is required before this method should be used in live donors.

CHAPTER 6: GENERAL DISCUSSION

This thesis has explored the polymeric gel forming mucins, the structural components of mucus in equine airways, with specific reference to the equine asthma-like disease, RAO. In health, the mucus layer lining the respiratory epithelium is beneficial, keeping it moist and offering protection as part of the innate immune system. As we have reviewed, there is more mucus in RAO horses airways compared to healthy horses, both whilst symptomatic and asymptomatic. This excess mucus in the airways contributes to pathology, blocking the small airways and contributing to impaired gas exchange. Therefore the aim of this PhD was to investigate mucins and their production sites in RAO horses so that we can better understand the disease and what occurs changing a protective element in health into a pathological one in disease.

Mucins are produced in mammalian airways in epithelial goblet cells and deeper sub-mucosal glands; however the extent and distribution of this in the equine airways was largely undetermined prior to work undertaken as part of this thesis. We were also unaware of the secreted mucin profile of RAO horses, when both asymptomatic and symptomatic. Much of the work of this thesis has been dedicated to addressing these unknowns.

6.1 RAO HORSE MUCUS AND MUCINS

We still do not fully understand why there is an excess of airway mucus in horses with RAO. There is some evidence in the literature that mucus in RAO horses has reduced clearance (Gerber *et al.*, 2000), though our understanding of the reasons behind the reduced clearance is limited. It could be that alterations in the mucins that are the structural components of mucus, be that by a change in their relative amounts or changed biochemical structures leads to altered physical properties and reduced clearance; or it could be an increased secretion of mucins is sufficient to alter mucus clearance in RAO airways, either by molecular interactions in the mucus gel making the gel more viscous and difficult to clear, or by the sheer volume of mucins being secreted overloading the ability of the mucociliary escalator. We know from studies on human airway diseases where mucus is part of the pathology, that there can be an increase in the amount of both of the major secreted mucins (MUC5B and MUC5AC), that the relative amounts of each of the mucins can change, and that the relative amounts of the glycoforms of a mucin can be different to mucus from healthy airways (Fahy, 2002; Groneberg *et al.*, 2002a; Kirkham *et*

al., 2002; Thornton *et al.*, 1991). Therefore we predicted that all 3 elements, secretion, relative amounts and structural changes, may be contributors to the mucus accumulation seen in RAO airways.

The mucins Muc5b and Muc5ac had previously been identified as the major mucins of healthy equine airway mucus by gene expression and glycoprotein identification, with Muc5b predominating, as apposed to MUC5AC predominating in healthy human airways (Gerber *et al.*, 2003; Rousseau *et al.*, 2011b; Rousseau *et al.*, 2007; Thornton *et al.*, 2008). However prior to the work undertaken in this thesis, less was known regarding the mucins in RAO horse mucus. We were aware that there was an up-regulation of *Muc5ac* gene expression, and that *Muc2* expression had not been identified when looked for (Gerber *et al.*, 2003); however no work on the gene expression for *Muc5b*, the predominant mucin of healthy equine airways, or indeed identification of any mucin glycoproteins in airway secretions from RAO horses had been undertaken.

It was our aim to determine what the predominant mucins and their relative amounts in RAO mucus are, both during disease exacerbation and when horses are asymptomatic. We have been able to establish, by mucin identification by immunoblotting and Western blotting after agarose gel electrophoresis, and by mass spectrometry identification of tryptic peptides, that, as in healthy equine airways, Muc5b and Muc5ac are the structural mucins of RAO mucus. Furthermore, we have shown, by immunodetection and identification of peptides on mass spectrometry, that Muc5b is the predominant mucin in RAO horse mucus when symptomatic and asymptomatic, as is the case in healthy horse airways. However, we have not been able to fully elucidate the answers to the questions in this thesis, due to lack of control of multiple variables (for example genetic heterogeneity, disease status and environmental factors affecting donors) and also due to problematic sampling techniques (to do with volume and dilution of mucus sample retrieved), leading to incomplete mucus sampling and inconsistencies of samples between donors. There undoubtedly will be differences in the mucin content of mucus between individual RAO horses, due to influences linked to both genetic and environmental factors, an area for future research, but something that we were unable to ascertain in this study.

RAO has been likened to human asthma, however our findings here highlight a difference between the human and equine diseases; in man the predominant mucin in health is MUC5AC, whilst in asthma MUC5B is the major mucin (Groneberg *et al.*, 2002a; Sheehan *et al.*, 1999; Sheehan *et al.*, 1995); whilst in horses there is no switch of the

relative amounts of the two mucins and Muc5b is the predominant mucin in both healthy airways and in RAO mucus. In both asthma and RAO though, MUC5B/ Muc5b is the predominant mucin in airway mucus.

In human asthma, mucins have been identified with an abnormal size and morphology (Sheehan *et al.*, 1999), and so an aim of this thesis was to identify if such changes exist in mucins isolated from RAO mucus. On examination of mucin size distribution we could not identify any size or morphological differences between RAO and healthy horse mucins, finding them to be of similar size distribution whether from exposed or not-exposed RAO and healthy control horses. However, the abnormal mucins identified from human asthmatic patients were retrieved from mucus plugs within the lungs, and we have not had access to such samples from equine airways to be able to investigate the biochemical propertied of such mucins. We also have to bear in mind that we were limited by a small sample size and that we found a lack of detectable mucin in mucus samples from control horses.

Two glycoforms, one high and one low charge form, of MUC5B have been reported in human airways mucus, with the low charge glycoform predominating in asthmatic mucus samples, therefore we were interested to see if this was true for RAO mucins. A faster migrating species of Muc5b, likely to be a higher charge glycoform, was identified in some of the mucus samples in this project, including samples from RAO horses but also from one control horse. The faster migrating band was evident when there was a stronger Muc5b signal in the sample, so it is plausible that we are only detecting the second species when there is overall more Muc5b in the sample. Or it could be that horses that produce more Muc5b are making 2 different glycoforms, compared to other horses in which just one glycoform predominates.

In our original hypothesis we said that it was likely that there would be an increased amount of both mucins correlating with the increased amount of endotracheal mucus found in RAO horses compared to healthy horses. Whilst we were able to confirm an increased detection of both Muc5b and Muc5ac in RAO horse mucus compared to control horse mucus, we were not able to confirm a positive correlation with the endotracheal mucus score due to potential limitations in the mucus collection methods employed. We found that TW sampling was more useful than BAL sampling to provide airway mucus samples with mucin that could be detected using our currently employed techniques; however we still found that the mucin content of TW samples did not correlate well with the amount of mucus visualised in the horses' large airways. Looking at

previous published data we find that TW mucus samples have previously been shown to have a positive correlation between mucin content and endotracheal mucus accumulation score in horses (Rousseau *et al.*, 2011b). However, we must bear in mind that the previous study examined a much larger sample size, that the majority of the horses had low endotracheal mucus scores, none of the horses examined had RAO, and a different, more simple mucus accumulation score of 0-3 was employed. The mucus from RAO horses, which we know has altered clearance properties, may be more difficult to obtain a representative sample by TW compared to mucus samples from healthy and IAD horses.

6.2 AIRWAY MUCIN CELL DISTRIBUTION IN HEALTH AND RAO

It was previously unknown exactly where in the horse airways the mucins Muc5b and Muc5ac, identified as the major structural components of airway mucus, were produced, both in health and disease. In this thesis we first determined the distribution of mucin producing cells in the healthy horse airways and then subsequently went on to examine the distribution of mucin cells in RAO horse airway, making comparisons between exposed and not-exposed environmental conditions for healthy and RAO horses.

In human airways the mucins MUC5B and MUC5AC are largely produced in 2 separate sites (surface goblet cells and glandular mucous cells), however, preliminary data from our laboratory had identified that this was not the case in equine airways, and we wanted to confirm and further quantify this. Data on the distribution of mucin-producing goblet cells in the equine trachea were previously scant, as was information on mucin cell distribution in the airways of both healthy and RAO horses (Pirie *et al.*, 1990a; Widdicombe and Pecson, 2002). It is not fully known if the increases of mucus in RAO airways is due to an increased size, number or secretory activity of mucin producing cells, whether this involves the epithelial goblet cells or sub-mucosal glands or both, or a combination of all of these factors. We have confirmed that mucins, notably Muc5b, are present in epithelial goblet cells and sub-mucosal glands of healthy and RAO equine airways, throughout the trachea and bronchi. We confirmed also that there is goblet cell hyperplasia during exacerbation of RAO.

A large inter-horse variation of goblet cell number per length of epithelium had been previously identified (Bartner *et al.*, 2006; Lugo *et al.*, 2006). We confirmed that this variation exists not only in healthy horses but also in RAO horses. However we also found that the size of goblet cells does not vary significantly in any one individual horse

along the length or around the circumference of the trachea, or between the trachea and the bronchi.

We were able to ascertain differences in goblet cell number between RAO and control horses, and that different exposure states affect this. When not-exposed to a dusty stable environment, healthy control horses have more goblet cells per length of bronchial epithelium than asymptomatic RAO horses; yet during exposure causing symptomatic RAO, the picture changes, with RAO-diseased horses having more goblet cells per length of tracheal and bronchial epithelium than healthy exposed controls. We also noted that all horses (RAO and healthy control) housed in a dusty stable environment have more goblet cells per length of bronchial epithelium than in their tracheal epithelium. Thus, exposure to a dusty environment would seem to lead to goblet cell hyperplasia in the bronchi of healthy horses, and to a greater extent, of the trachea and bronchi in RAO horses. We found smaller goblet cell size in exposed horses in the bronchi of control horses and the tracheas of RAO horses. We hypothesised that the smaller goblet cell size noted during exposure could be due to increased secretion of mucins, although why there is an anatomical difference between healthy control and RAO horses is unclear. It could be that in RAO horses there is an altered pattern of mucin secretion, affected by the inflammatory processes involved in symptomatic RAO.

In the healthy horse airway, we found that the mucin positive sub-mucosal gland size was larger in the trachea compared to the bronchi, with a larger percentage of the tracheal glands staining positive for mucin than the bronchial glands. Subsequently we also found this to be true in horses suffering from RAO. We discovered that exposed RAO horses have larger areas of mucin-positive sub-mucosal gland than their exposed healthy control counterparts. Unfortunately we do not have complete data on mucin content of sub-mucosal glands in not-exposed control horses to be able to compare with asymptomatic RAO horses.

We were able to successfully produce Muc5b-specific antisera for use in immunohistochemistry, however unfortunately it was not possible to prepare Muc5ac-specific antisera. Therefore we have valid data for Muc5b content of equine airway mucin cells, and can make useful comparisons with the general mucin stain (PAS AB), although we are unable to draw conclusions about the distribution of Muc5ac producing cells. We found that Muc5b is produced both in the epithelial goblet cells and the sub-mucosal glands in healthy and RAO diseased horses. We did find some discrepancies existed between the general mucin stain and Muc5b, and that this was not consistent in different

areas of the respiratory tract, indicating that another mucin may well be contributing to the general mucin stain as well as Muc5b in some locations more than others. Specifically we found that the area for the general mucin stain was significantly larger than Muc5b stain area for goblet cells but not sub-mucosal glands, leaving us to speculate that Muc5ac accounts for this discrepancy. It is interesting that we found this difference to be variable between horses, so perhaps the ratio of Muc5b: Muc5ac in epithelial goblet cells varies between individuals. Conversely, we found that in the bronchial sub-mucosal glands, Muc5b stain area was larger than that for the general mucin stain, and this may reflect either measurement errors or a potential increased sensitivity of the Muc5b antibody staining compared to the general mucin stain. If that is true, then this could indicate that perhaps there is less Muc5ac or other mucin(s), than Muc5b in the bronchial sub-mucosal glands. To be able to investigate this thoroughly, a reliable, specific antibody for Muc5ac for immunohistochemical use needs to be developed and tested on serial sections of the same tissues.

From our above findings, the increased amounts of airways mucus in symptomatic RAO horses can be attributed to a number of factors, including an increase in number (hyperplasia) of both tracheal and bronchial epithelial goblet cells; and possibly also an increased secretion from these cells, indicated by a smaller goblet cell size in exposed horses. RAO horses also have a larger sub-mucosal gland size than healthy controls, at least during exposure to a dusty stable environment, which will also be a factor contributing to the secreted mucus in the airways of symptomatic RAO horses. As for asymptomatic RAO horses, we know that they also have increased endotracheal mucus compared to healthy controls, but we have not been able to definitively conclude the source of the excess mucus in theses horses. We actually found that not-exposed control horses have more goblet cells per length of epithelium than asymptomatic RAO horses. It follows logically that the source of excess mucus seen in asymptomatic RAO horse airways must be the sub-mucosal glands. However we do not have complete data available for sub-mucosal glands of not-exposed control horses, specifically, we are missing the tracheal gland data. The data we do have for comparison of mucin-positive sub-mucosal gland size between not-exposed RAO and control horses shows no significant difference for the bronchial glands. Therefore if the source of excess mucus in asymptomatic horses is attributable to mucins, they must be being secreted largely from the tracheal sub-mucosal glands, which are larger than the bronchial glands. It is most unfortunate that we

did not have access to tracheal tissue from healthy control horses in the tissue bank to be able to confirm this hypothesis.

6.3 HARVESTING EQUINE AIRWAY EPITHELIUM FROM LIVE RAO DONORS FOR ALI CLUTURE

The aim of the final experimental chapter of this thesis was to develop a rigorously controlled and physiologically relevant experimental model to enable us to study mucin production in epithelial cells from RAO horses, so that we can investigate and manipulate the factors that might lead to increased mucin production and secretion. The ALI culture model developed for human bronchial epithelium mimicking the *in vivo* airway epithelium has previously been adapted and successfully developed using equine airway epithelium, (Abraham *et al.*, 2011; Oslund *et al.*, 2010; Quintana *et al.*, 2011; Schwab *et al.*, 2013; Schwab *et al.*, 2010). However, our eventual aim is to study mucin production in RAO horse epithelium, and we have encountered difficulties in obtaining a source of post mortem fresh RAO airways for tracheal digestion and harvest of epithelium for the culture model. We therefore wanted to test and develop a minimally invasive method of harvesting cells from live donors, so that we could feasibly collect epithelium cells and grow them in ALI without necessitating the death of the RAO donor.

We encountered difficulties in development of the cell harvesting and ALI culture technique, the most frequent of which was fungal contamination of cells, but we also experienced fibroblast contamination of epithelial cultures, and bacterial infection of cells. As fungal spores are commonly encountered in a horse's environment and often present in horse airways, fungal contamination of primary cell lines is likely to be a perpetual problem that will be encountered. We experienced failure of cells from certain donors to adhere and proliferate, and we are unsure as to why this happened, but it potentially could be due a combination of genetic variation, donor age, and environmental factors. We have encountered this problem previously, in earlier work with equine airway cells in our laboratory (K. Rousseau, University of Manchester, personal communication). Other difficulties that we faced with cell culture were the expense and time involved getting to the differentiated ALI stage, and that once ALI had been achieved, the cultures were not of a satisfactory quality in that they did not form a tight epithelial layer, and therefore not representative of the *in vivo* situation. Positive outcomes that we can take away from this experimental investigation is that under some circumstances it was possible to yield

sufficient cell numbers by tracheal brushing to enable ALI culture, and we confirmed that cells require propagation after collection before seeding onto the ALI transwell inserts. We also had occasional success in freezing cells collected by tracheal brushing method, which could later be taken to ALI, if they were frozen in at least 20 % FBS.

A group of researchers have recently reported a technique of bronchial biopsy in the live horse for harvesting equine airway cells for culture (Frellstedt *et al.*, 2013). However, they have only used the cells in submerged culture, not ALI culture, and have not yet fully published their data or reported the percentage of horses from which biopsies are successfully taken to cell culture. This biopsy technique, if successful, may be another useful way to harvest cells from live equine donors and take them to ALI culture.

Aside from the numerous technical problems outlined above that we have encountered in trying to set up ALI cultures, we must also remember that the ALI set up is designed to study mucin production of epithelial goblet cells and does not provide a platform for sub-mucosal gland cell mucin production. We have identified the sub-mucosal glands as an important source of secreted mucin contributing to increased endotracheal mucus, at least in exposed RAO horses, and also most likely in asymptomatic RAO horses. Therefore, while an ALI culture model is still desirable and will be useful for studying the factors affecting mucin secretion and also to examine the biochemical properties of secreted mucins under different conditions, it is likely it will not provide all of the answers regarding increased mucin secretion and increased airway mucus in RAO horse airways.

6.4 FUTURE WORK

In this thesis we have explored the mucins present in RAO horse mucus and their production sites in the equine airways, in symptomatic and asymptomatic horses and compared to healthy control horse samples. Several shortcomings of these investigations automatically call for further detailed studies in the field, in order to be able to fully elucidate the answers we are looking for.

One problem we encountered was that we were unable to obtain representative retrieval of mucus samples from RAO horse airways, and so development of a standardised method of mucus collection from horse airways would yield samples from

which quantitative data could be gained. Use of tubing of a larger diameter, ideally without the use of saline (which dilutes the samples) may help to achieve this. The other problem we face when sampling even from an experimental herd of horses that have their environment controlled is the phenotypic and genetic variation between donors, which tend to be of a variety of horse breeds, ages and gender. In this vein, one way that we have tried to address this is by production of a cell culture model and so it is worth persisting further with cell culture models to produce mucin secretion from a standardised controllable source. If we can improve the culture technique to minimise problems and reliably grow cells to ALI from live donors using minimally invasive sampling technique, this will prove very useful for future research on mucin production in RAO.

One area of our investigation on the production of mucins in the equine airways that requires further work is the development of a reliable Muc5ac antiserum for use in immunohistochemistry, so that we can complement the data set already in existence and answer some of the questions remaining over the two major mucins identified in equine airway mucus, Muc5b and also Muc5ac, and their production sites. Other problems that we encountered during our histological investigations were that we did not have tracheal mucosa samples from controlled not-exposed horses and this data are really needed to confirm our suspicions regarding the tracheal sub-mucosal glands being a major source of Muc5b in asymptomatic RAO horses.

Through whatever means mucins are collected from RAO airway cells in the future, a thorough understanding of mucin biochemistry, with experiments to help our understanding of what controls mucin cell production and secretion in health and disease will guide us to the formulation of effective therapeutic targets in the long term.

6.5 CONCLUSION

Our investigations on mucus and mucins in RAO have identified that horses with RAO have more endotracheal mucus than healthy controls, and that Muc5b and Muc5ac are the major mucins present in RAO horse mucus, both during symptomatic disease and when horses are asymptomatic. We have also identified that, as it the case in the healthy horse, Muc5b is the predominant mucin in RAO horse airways.

We can also conclude that mucins are produced in epithelial goblet cells and sub-mucosal glands dispersed throughout the length and circumference of the equine trachea

and bronchi. There is a great variation between horses in the number of epithelial goblet cells, and also the size of sub-mucosal glands; however goblet cell size is less varied between healthy individuals. Goblet cell hyperplasia occurs in symptomatic exposed RAO horse airways, although goblet cells are smaller than in asymptomatic RAO horse airways. Exposure to a dusty stable environment is associated with more goblet cells per length of bronchial compared to tracheal epithelium in all horses. RAO horses have larger sub-mucosal glands containing more mucin than control horses, and the tracheal glandular secretions are likely to be a major source of mucin providing the structural framework of the increased mucus noted in symptomatic and asymptomatic RAO horse airways.

It is possible to harvest equine tracheal epithelial cells by brushing to enable fresh primary culture and development of ALI culture. It is also possible to freeze cells collected via tracheal epithelial brushing in 20 % FBS and then culture to ALI at a later date. The techniques employed for cell harvesting and ALI culture however require refinement before meaningful results can be obtained if we are to better understand the role of mucins and their contribution to pathology in equine RAO.

APPENDIX I

Table AI.1 **CLINICAL RAO PATIENT (RAO.CD.1) DETAILS**. The table shows signalment data for the RAO-diseased horse (RAO.CD.1) from which tracheal wash (TW) and bronchoalveolar lavage (BAL) samples were provided for *Chapter 3.2.1*. Details of BAL fluid cytology findings are shown including: subjective mucus score on a scale of 0 (none) to 4 (profuse amount of mucus on slide); differential nucleated cell count percentage of neutrophils (No), macrophages (Mo), and lymphocytes (LC). Gelding (G).

				BAL Cytology			
Horse ID	**Age (years)**	**Sex**	**Breed**	**Mucus**	**No**	**Mo**	**LC**
RAO.CD.1	10	G	Cob	4	63%	29%	8%

Table AI.2 **CLINICAL RAO PATIENTS (I - XIV) DETAILS**. The table shows age, where known for the RAO-diseased horses from which tracheal wash (TW) or bronchoalveolar lavage (BAL) samples were provided for *Chapter 3.2.1*. Details of TW cytology findings are shown including: total nucleated cell count (TNC); subjective mucus score on a scale of 0 (none) to 4 (profuse amount of mucus on slide); subjective assessment scores, on a scale of 0 (none) to 4 (profuse numbers), are given for proportionate cellular numbers of dust (du) or vacuolated (v) macrophages (Mo); active (a) or degenerate (d) neutrophils (No); lymphocytes (LC); eosinophils (Eo); fungal hyphae (FH) and fungal spores (FS) observed on cytology. Details of BAL differential nucleated cell count percentages are shown, including inflammatory cells and epithelial cells (EC).

			TW Cytology Observations										BAL Differential Cell Count				
Horse ID	**Age (years)**	**TW or BAL**	**TNC**	**mucus**	**Mo (du)**	**Mo (v)**	**No (a)**	**No (d)**	**LC**	**Eo**	**FH**	**FS**	**No**	**LC**	**Eo**	**EC**	**Mo**
I		BAL											41%	33%	0%	6%	20%
II	13	TW	0.4	2	2	2	2	0	1	0	0	0					
III	14	TW	82.2	3	3	3	4	2	1	0	0	0					
IV	10	TW	0.2	4	4	3	3	0	1	0	0	2					
V		TW	1.4	2	3	3	2	0	1	0	0	2					
VI		TW	0.8	3	3	2	4	2	1	0	1	3					
VII		TW	8.1	3	1	2	4	2	1	0	1	0					
VIII	9	TW	8.2	3	4	1	3	1	1	0	0	3					
VIX		TW	13	1	3	3	4	1	1	0	0	2					
X	15	TW	1.1	1	3	2	3	1	1	0	2	2					
XI		BAL											62%	19%	0%	7%	12%
XII	9	TW	9.6	3	2	3	2	0	1	0	1	3					
XIII	7	TW	3.9	3	3	3	2	0	2	0	0	3					
XIV	12	TW	0.1	3	4	3	2	0	2	1	0	4					

Table AI.3 **EXPERIMENTAL HERD RAO HORSES (1 - 10) DETAILS**

The table shows signalment details for the RAO-diseased horses from which tracheal wash (TW) samples were provided for *Chapter 3.2.2*. Horses were either asymptomatic (Asympt) or symptomatic (Sympt) at time of mucus collection. Breeds of horse included: Quarter horse (QH), Belgian Warmblood (B WB), Percheron (Per), Thoroughbred cross (TB X), Appaloosa (App), Cross-breed (X), Canadian (Can), and Standardbred (SB) breeds. Mare (M), Gelding (G).

Horse ID	Group	Age	Sex	Breed
1	Asympt	26	G	QH
2	Asympt	20	M	B WB
3	Asympt	18	M	QH
4	Asympt	20	M	Per
5	Sympt	21	M	TB X
6	Sympt	16	G	App
7	Sympt	12	M	TB X
8	Sympt	20	M	X
9	Sympt	12	M	Can
10	Sympt	8	G	SB

Fig. AI.1 **OWNER INFORMED CONSENT INFORMATION SHEET**. The information sheet given to owners of RAO horse patients to ask permission for informed consent to use the mucus samples as part of this thesis is shown below. Owners were read this information by the veterinary surgeon performing the disease investigation and then had to sign a consent form (*Appendix I Fig. A1.2*) before samples could be used in this project.

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Examination of mucus properties in horses suffering from Recurrent Airway Obstruction (R.A.O. or "heaves"): Owner Information Sheet

Dear horse owner

The University of Liverpool Veterinary School and The University of Manchester are currently undertaking a study sponsored by the Horserace Betting Levy Board (H.B.L.B.) looking at the properties of mucus from airways of horses suffering from Recurrent Airway Obstruction (R.A.O., also known as C.O.P.D., "heaves", or "broken wind"). As part of this, you and your horse are invited to take part. We would be very grateful if you would allow us to take some samples from your horse and ask you some questions.

Please read the following information carefully and please ask if you would like more information or if there is anything you do not understand, my contact details are provided at the end of this letter. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to. If you decide not to participate this will **not** affect the veterinary treatment of your animal.

Why are we getting these samples?
R.A.O. is a common disease of horses that is poorly understood. Part of the pathology of the disease involves an increased amount of mucus production in the airways. Little is known about the properties of the mucus that is produced and how this impacts on the disease state of the affected horse. We are trying to investigate if the mucus properties in horses with R.A.O. are different from normal horses. We will do this by analysing the chemical properties of the mucus produced in the airways of normal and diseased horses. This will allow us a greater understanding of how excess mucus is produced in R.A.O. and hopefully can lead to the development of new ways to combat the problem. This is especially important, as R.A.O. is usually a life-long condition and can be difficult to treat and manage.

What samples are we collecting?
For this study we are interested in obtaining samples from horses respiratory tracts, including mucus collected from the trachea and small airways (bronchi). Your vet will be taking the same samples for diagnostic purposes in your horse, and we will simply take the excess material collected. We will then analyse the samples in our labs to see what the properties of the mucus and the cells producing them are. No samples will be taken from your horse purely for research purposes without a clinical reason. If your vet does not consider any of the samples necessary for helping to diagnose or monitor your horse's condition, then they will not be taken.

What does this involve?
Your vet will perform endoscopy to examine your horse's trachea (windpipe). Your horse may or may not require sedation for this procedure. Your vet may also pass the endoscope further into the

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small airways to examine deeper within the lungs. During the procedure your vet will examine the airways for presence of inflammation and mucus. Your vet will add some sterile fluid via the endoscope into the trachea and then suck the fluid out to allow collection of a sample of mucus from the trachea. This is called a tracheal wash. Your vet may also repeat this procedure in the small airways to collect a sample of the mucus produced there, this is called a broncho-alveolar lavage (B.A.L.). Your vet will submit the samples to a laboratory for analysis and will inform you of the results when they are available. Your vet will allow us to access any excess to samples that are not needed by the laboratory.

Anything more involved?
We may like to know a bit about your horse and its disease; if this is the case we might like to contact you in the future with a short questionnaire. Please indicate on the consent form if you would prefer to not be contacted.

Further information
Samples and information obtained from the questionnaire may be retained for up to six years and possibly used in future projects. All data will be stored in a secured database only accessible by people working on the project. If you decide you want to withdraw from the study you may do so without explanation, and any information you have given can be destroyed.

All answers from the questionnaire will be kept strictly confidential. Results from the study will be printed in veterinary journals and also in the non-veterinary animal press, but you and hour horse will not be identifiable from any published work.

What next?
If you are happy to allow your horse to become involved, then please read and sign the consent form, and we can start getting the samples. Please note, unfortunately due to the large number of samples, we will not be able to give you back any individual results from your horse but your vet will inform you about the laboratory results they obtain.

Many thanks,

Adele Williams BVSc MRCVS
Faculty of Life Sciences, Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, The Michael Smith Building, Oxford Road, Manchester, M13 9PT.

If there are any problems, please let us know by contacting Adele Williams or Peter Clegg via email: adelew@liv.ac.uk, pclegg@liverpool.ac.uk, and we will try to help. If you remain unhappy or have a complaint which you feel you cannot come to us with then you should contact the Research Governance Officer on 0151 794 8290 (ethics@liv.ac.uk). When contacting the Research Governance Officer, please provide details of the name or description of the study (so that it can be identified), the researcher(s) involved, and the details of the complaint you wish to make.

Information sheet version 2: 08/10/09

Fig. AI.2 **OWNER INFORMED CONSENT FORM** The consent form given to owners of RAO horse patients to sign, giving permission by informed consent to use the mucus samples as part of this thesis is shown below. Owners were read an information sheet (*Appendix I Fig. AI.1*) by the veterinary surgeon performing the disease investigation prior to signing this consent form before samples could be used in this project.

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CONSENT FORM

Title of Research Project:	Examination of mucus properties in horses suffering from Recurrent Airway Obstruction (R.A.O. or "heaves").	
Researcher(s):	Miss Adele Williams, Professor Peter Clegg, Dr David Thornton.	**Please initial box**

1. I confirm that I have read and have understood the information sheet dated [08/10/09] for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I am willing to be contacted in the future for questionnaire participation. ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected. ☐
4. I understand that, under the Data Protection Act, I can at any time ask for access to the information I provide and I can also request the destruction of that information if I wish. ☐
5. I agree to take part in the above study. ☐

Participant Name	Date	Signature
Name of Person taking consent	Date	Signature
Adele Williams Researcher	Date	Signature

The contact details of lead Researcher (Principal Investigator) are:

Adele Williams: Telephone: 07795515203,
Email: adele.williams@postgrad.manchester.ac.uk
adelew@liv.ac.uk
Faculty of Life Sciences,
Wellcome Trust Centre for Cell-Matrix Research,
University of Manchester,
The Michael Smith Building,
Oxford Road, Manchester, M13 9PT.

[Version Number 1] Mucus properties in R.A.O. 1
[08/10/09]
[AW]

Table AI.4 **EXPERIMENTAL HERD RAO AND CONTROL HORSES (11 - 22) DETAILS**. The table shows signalment details for the RAO-diseased horses from which tracheal wash (TW) mucus samples were provided for *Chapter 3.2.3*. Horses were either healthy controls or suffering from RAO. Breeds of horse included: Cross-breed (X), Thoroughbred (TB), Standardbred (SB), Quarter horse (QH), Alezan (Alez) and Palomino (Pal) breeds.

Horse ID	Group	Age	Breed
11	Control	19	X
12	Control	11	TB
13	Control	16	SB
14	Control	13	SB
15	Control	20	QH
16	Control	15	SB
17	RAO	23	Alez
18	RAO	19	Pal
19	RAO	26	QH
20	RAO	23	X
21	RAO	21	QH
22	RAO	18	QH

Table AI.5 **EXPERIMENTAL HERD TISSUE BANK DONORS RAO AND CONTROL HORSES (TB-1 TO TB-19) DETAILS**

The table shows signalment details (*at time of euthanasia) for the RAO-diseased and control experimental herd horses that were donors for the tissue bank samples used in *Chapter 4*. Horses were either healthy controls or suffering from RAO, and had been housed either not-exposed or exposed to a dusty stable environment at the time of euthanasia. Breeds of horse included: Standardbred (SB), Quarter horse (QH), Thoroughbred (TB), Arab, Morgan (Morg), pony and cross (X) breeds. Mare (M); gelding (G). Tb- followed by number denotes individual tissue bank horse identification (ID).

Horse ID	Group	Exposure status*	Age*	Sex	Breed
Tb-1	Control	Not-exposed	16	M	SB
Tb-2	Control	Not-exposed	23	M	QH
Tb-3	Control	Exposed	13	M	SB
Tb-4	Control	Exposed	14	M	SB
Tb-5	Control	Exposed	13	M	TB
Tb-6	Control	Exposed	29	G	QH
Tb-7	Control	Exposed	13	G	Arab
Tb-8	Control	Exposed	20	G	Morg
Tb-9	RAO	Not-exposed	17	G	Arab X
Tb-10	RAO	Not-exposed	23	M	X
Tb-11	RAO	Not-exposed	14	M	QH
Tb-12	RAO	Not-exposed	19	M	QH X
Tb-13	RAO	Not-exposed	14	M	X
Tb-14	RAO	Exposed	15	M	QH
Tb-15	RAO	Exposed	20	M	SB
Tb-16	RAO	Exposed	23	G	QH X
Tb-17	RAO	Exposed	21	G	QH X
Tb-18	RAO	Exposed	20	G	Pony
Tb-19	RAO	Exposed	13	M	QH X

Fig. AI.3 **HISTORICAL LUNG FUNCTION DATA FROM EXPERIMENTAL HERD RAO HORSES 1 - 10**. Lung function testing data on these RAO horses are shown below. These data were previously obtained by our collaborators, Dr. J.P. Lavoie and colleagues (University of Montreal), in earlier experiments not related to the current study, to confirm a diagnosis of RAO. A diagnosis of RAO is made if maximal change in pleural pressure (ΔPpl_{max}) is increased by more than 15 cm H_2O, accompanied by a BAL fluid (BALF) neutrophil percentage of over 25% when the horse is housed in an exposed environment (Robinson, 2001). These experimental RAO horses all show a measurable change in ΔPpl_{max} of more than 15 cm H_2O, which was also accompanied by > 25% neutrophils in BALF (data not shown) during exposure. Numbers and associated symbols in key refer to individual horses 1 - 10 from the experimental herd.

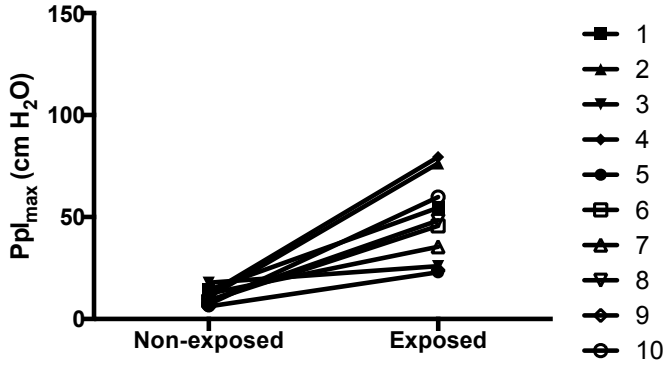

Table AI.6 **COLLECTION AND SOLUBILISATION OF TW MUCUS SAMPLES FROM RAO EXPERIMENTAL HORSES 1 - 10.** TW mucus samples were collected from experimental herd RAO horses 1 - 10 via trans-endoscopic infusion of 100 mL sterile 0.9 % saline and suction retrieval. Volume retrieved varied between individual horses as outlined below. An equal volume of 8 M GuHCl was added to each TW mucus sample in order to solubilise them.

Horse	Empty tube weight (g)	Tube weight with TW sample (g)	TW sample weight (g)	TW sample volume (mL)	Volume 8M GuHCl added (mL)	Solubilised TW sample volume (mL)
1	12.18	21.81	9.63	11.0	11.0	22.0
2	11.99	24.27	12.27	14.0	14.0	28.0
3	11.99	28.36	16.37	18.0	18.0	36.0
4	12.23	28.75	16.52	18.0	18.0	36.0
5	12.54	23.51	10.97	12.0	12.0	24.0
6	12.13	26.60	14.47	16.0	16.0	32.0
7	12.16	24.77	12.61	15.0	15.0	30.0
8	12.42	18.63	6.21	8.0	8.0	16.0
9	14.11	24.15	10.05	7.5	7.5	15.0
10	14.27	21.08	6.81	11.0	11.0	22.0

Fig. AI.4 **HISTORICAL LUNG FUNCTION DATA FROM EXPERIMENTAL HERD HORSES 11 - 22**. These data were previously obtained by our collaborators, Dr. J.P. Lavoie and colleagues (University of Montreal), in earlier experiments not related to the current study, to confirm a diagnosis of RAO. A diagnosis of RAO is made if maximal change in pleural pressure (ΔPpl_{max}) is increased by more than 15 cm H_2O, accompanied by a BALF neutrophil percentage of over 25% when the horse is housed in an exposed environment (Robinson, 2001). The experimental RAO horses all showed a measurable change in ΔPpl_{max} of more than 15 cm H_2O, which was also accompanied by > 25% neutrophils in BALF (data not shown) during exposure; whilst the control horses did not. **A**. Control horses. **B**. RAO horses. Symbols refer to individual horses as indicated by designated numerical assignment (11-16, control horses; 17-22 RAO horses) in key.

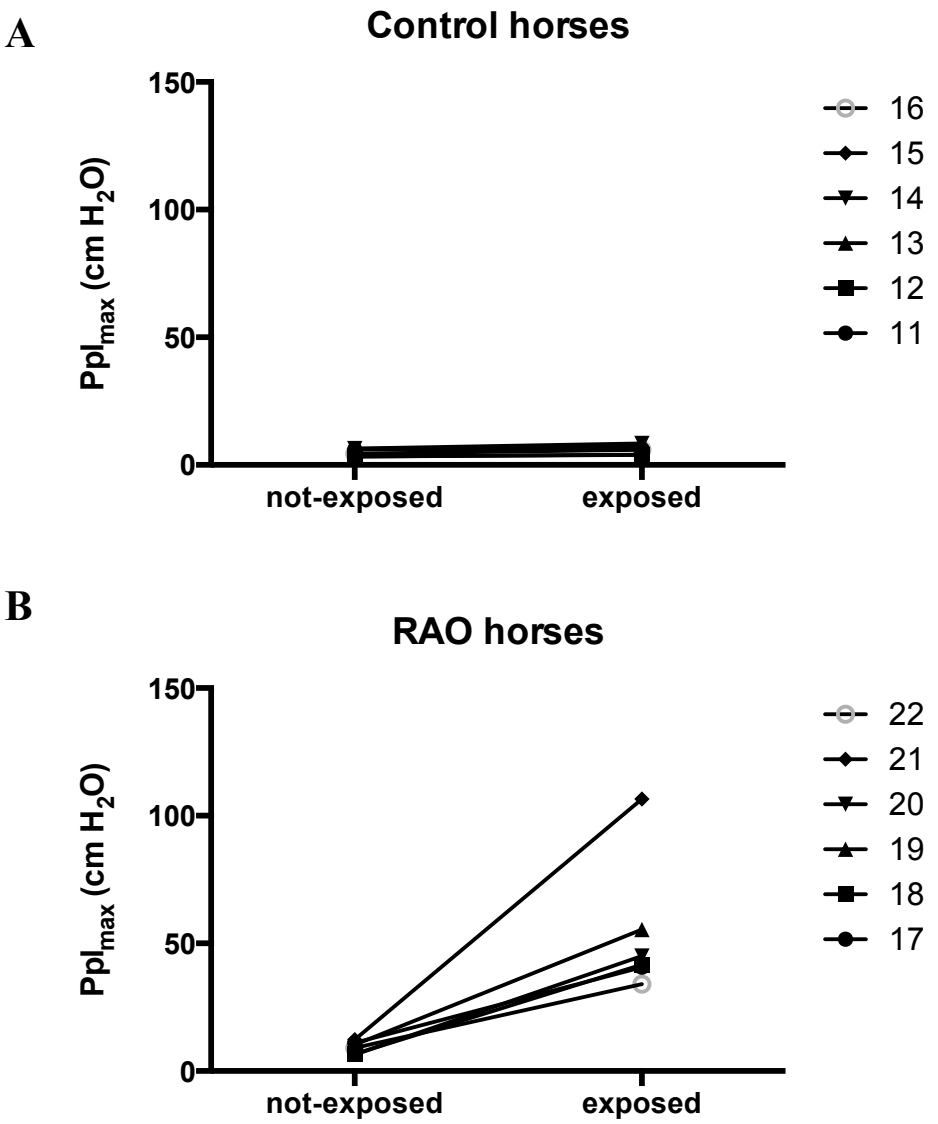

Table AI.7 **SOLUBILISATION AND EQUAL DILUTION OF TW MUCUS SAMPLES FROM RAO AND CONTROL EXPERIMENTAL HORSES 11-22 DURING NOT-EXPOSED AND EXPOSED ENVIRONMENTAL CONDITIONS.** Volume of 8M GuHCl required to solubilise TW mucus samples from experimental herd control and RAO horses before exposure and during exposure to an environment causing symptoms in the RAO horses. Volume of 8M GuHCl added to each sample to then bring all samples to an equal dilution (1:10.8; dilution of sample that required the highest volume of GuHCl to solubilise the sample).

Horse ID	Control (0), RAO (1)	Not-exposed (0), Exposed (1)	initial volume collected (ml)	Volume 8M GuHCl required to solubilise sample	Volume 8M GuHCl needed to make all samples equal dilution
11	0	0	22	22	193.22
12	0	0	26	26	228.35
13	0	0	27	32	232.13
14	0	0	21	26	179.43
15	0	0	22	22	193.22
16	0	0	20	30	165.65
17	1	0	26	31	223.35
18	1	0	26	136	118.35
19	1	0	26	221	33.35
20	1	0	26	219	35.35
21	1	0	27	222	42.13
22	1	0	22	87	128.22
11	0	1	25	25	219.57
12	0	1	26	26	228.35
13	0	1	24	29	205.78
14	0	1	20	25	170.65
15	0	1	21	26	179.43
16	0	1	19	34	151.87
17	1	1	19	34	151.87
18	1	1	25	238	6.57
19	1	1	23	225	0.00
20	1	1	25	80	164.57
21	1	1	21	101	104.43
22	1	1	17	27	139.30

Table AI.8 **ENDOTRACHEAL MUCUS ACCUMULATION SCORES FROM RAO AND CONTROL EXPERIMENTAL HORSES 11-22 DURING NOT-EXPOSED AND EXPOSED ENVIRONMENTAL CONDITIONS.** Video-endoscopy was used during mucus collection to enable endoscopic scoring of tracheal mucus accumulation. Seven observers each scored video footage of tracheal mucus accumulation on two separate occasions, one month apart; giving a total of 14 observations per mucus sampling time per horse. Observers were blinded to the identity of each horse. Tracheal mucus accumulation score for each observation for each individual horse is shown **A**. before (not-exposed) and **B**. after exposure to an environment that induces clinical signs of disease in RAO-affected horses. Numbers are identifiers for each individual horse where 11 - 16 are control animals and 17 - 22 are RAO-diseased horses.

A

Horse	Endotracheal mucus score individual observations: Not-exposed conditions													
11	0	2	2	1	4	2	2	1	2	0	1	3	1	1
12	1	1	1	0	2	1	1	1	0	0	0	2	1	2
13	0	0	1	0	1	1	1	1	0	0	0	1	1	1
14	1	3	1	0	0	0	0	0	1	0	0	1	0	0
15	0	0	0	0	0	0	0	0	0	0	0	2	0	0
16	0	0	0	1	2	0	0	0	0	0	0	1	0	0
17	2	2	2	2	3	3	2	2	2	1	2	3	3	3
18	3	2	2	4	4	3	2	2	3	2	3	4	3	3
19	2	3	3	4	4	4	3	3	3	0	4	4	4	3
20	2	3	3	1	4	4	2	4	4	1	2	3	3	4
21	0	2	1	0	1	1	1	0	1	0	2	1	1	5
22	2	2	3	3	3	4	3	3	3	3	3	3	3	4

B

Horse	Endotracheal mucus score individual observations: Exposed conditions													
11	0	1	1	0	2	1	2	0	2	0	1	2	1	0
12	0	0	0	0	1	0	0	0	0	0	0	1	0	0
13	1	1	2	1	3	3	3	1	3	1	2	2	3	3
14	0	1	2	0	1	2	1	1	1	0	0	1	2	0
15	0	0	1	0	0	0	0	0	1	0	0	1	1	0
16	0	1	0	1	1	0	1	0	0	0	0	2	0	0
17	4	3	4	4	5	5	4	4	4	4	4	3	5	5
18	5	5	5	5	5	5	5	5	5	5	5	4	5	5
19	4	4	5	4	5	5	4	5	5	4	4	4	5	5
20	2	2	3	2	4	4	3	2	3	2	3	2	4	4
21	3	4	4	3	5	5	3	2	4	2	3	3	4	4
22	1	1	2	1	2	3	2	3	3	1	1	2	2	2

APPENDIX II

Fig. AII.1 **MUC5B AND MUC5AC ANTIBODY PURIFICATION FRACTIONS: POSITIVE AND NEGATIVE CONTROL TISSUE TESTING**. Representative photomicrographs of formalin fixed equine salivary gland (SG) and stomach tissues with affinity purified fractions of equine mucin polyclonal antisera (eqMAN5ac-I, eqMAN5b-I). **A**. Muc5b antibody fractions **B**. Muc5ac antibody fractions. Slides made from serial sections of paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection and fast green (1 % w/v) counterstain. Numbers 1 - 6, 10 and 11 refer to affinity purification fractions as described in *Fig 4.1*. Red circles indicate examples of positive staining mucin tissue. Red boxes highlight Muc5b fractions used in future investigations. Scale bar 100 µm; original images captured at x 40 magnification.

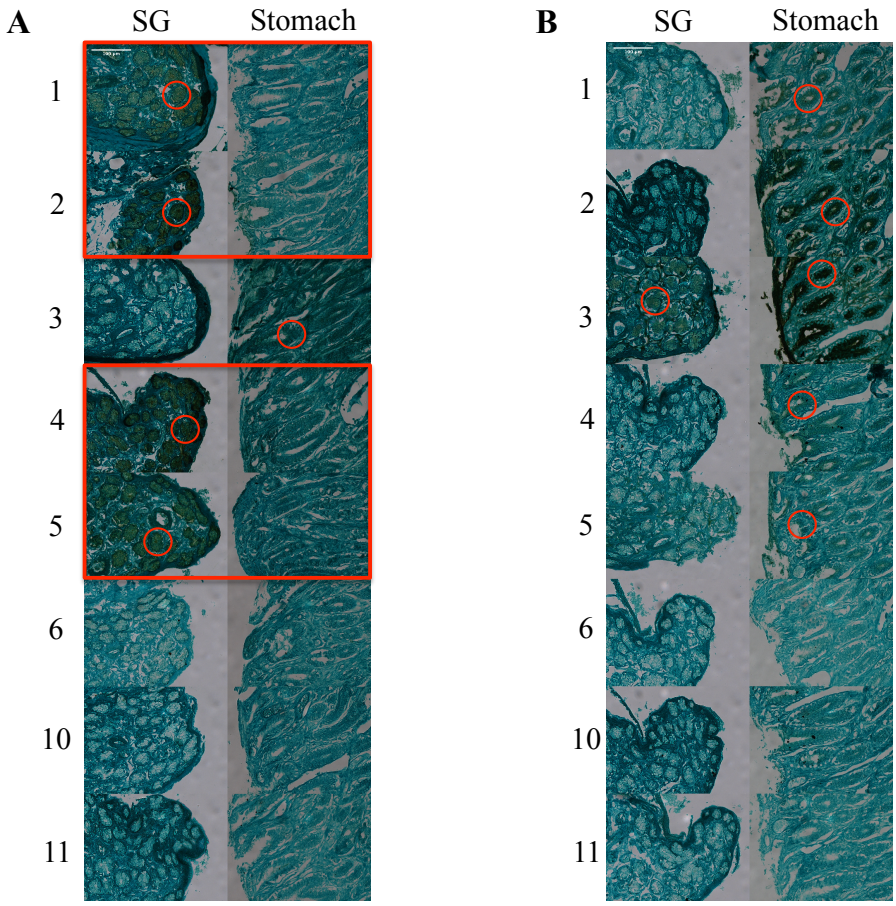

Fig. AII.2 **EQUINE MUC5B ANTIBODY AFFINITY PURIFICATION ELUTION COUNTERSTAIN METHOD.** Slides made from paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection. Salivary gland is positive control and stomach tissue is negative control for Muc5b. Images shown for Muc5b fractions 1, 2, 4, and 5 with fast green counterstain 0.5 % (w/v). Scale bar 200 µm; original images captured at x 10 magnification.

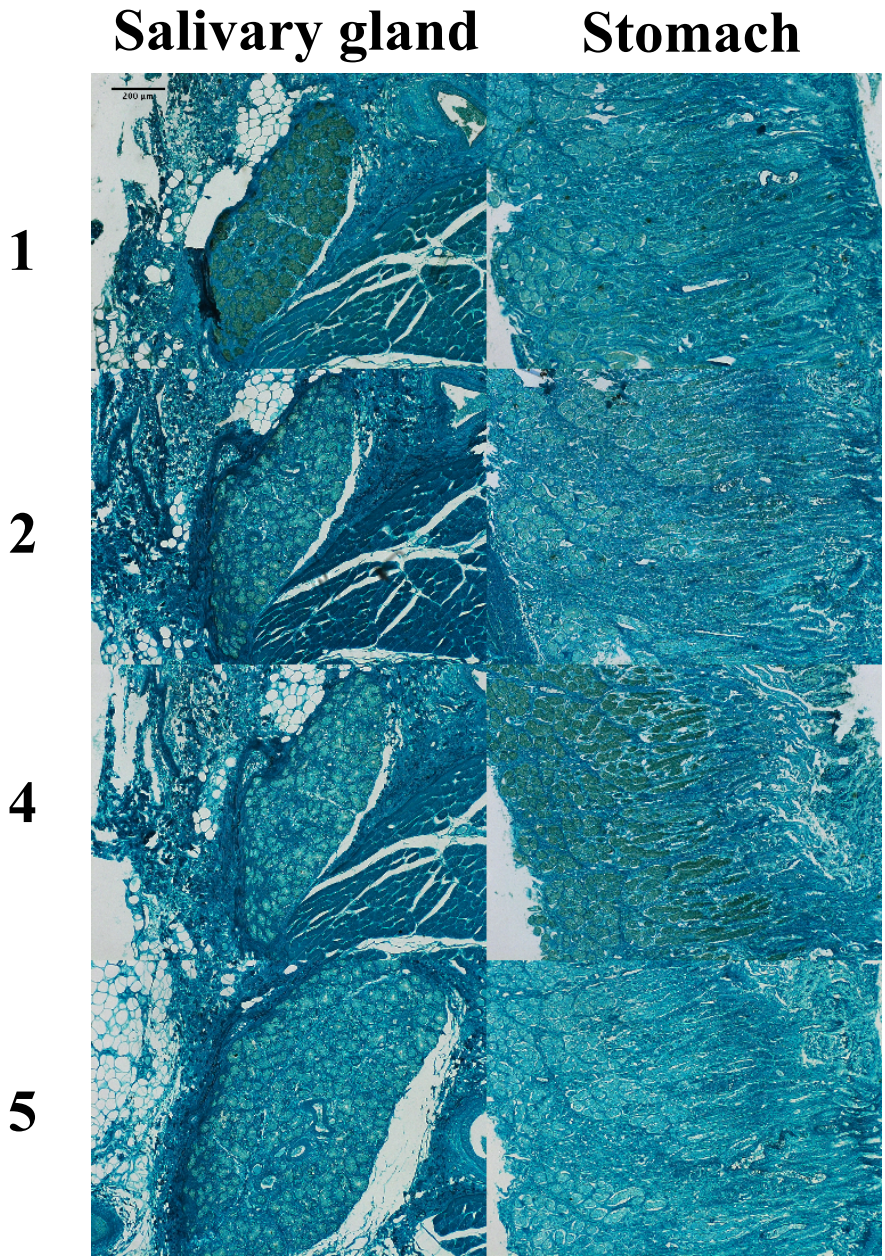

Fig. AII.3 **EQUINE MUC5AC ANTIBODY AFFINITY PURIFICATION ELUTION COUNTERSTAIN METHOD.** Slides made from paraffin-embedded tissue were stained using antibody at 1:100 dilution incubated overnight and detected using DAB colour detection. Salivary gland is negative control and stomach tissue is positive control for Muc5ac. Images shown for Muc5ac fractions 1, 2, 4, and 5 with fast green counterstain 0.5 % (w/v). Scale bar 200 µm; original images captured at x 10 magnification.

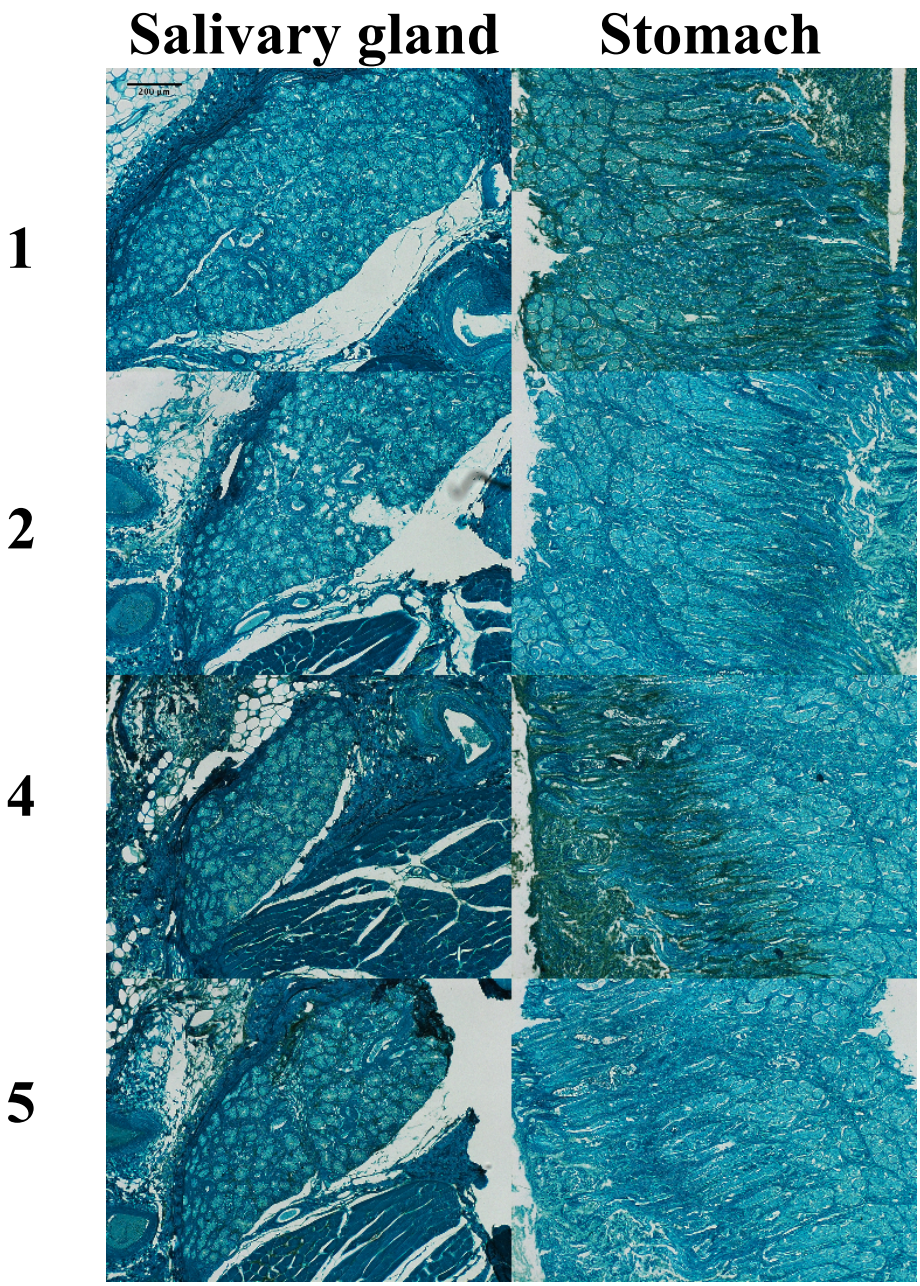

Fig. AII.4 **KERATIN BLOCKADE TO ELIMINATE BACKGROUND DAB STAINING: MUC5AC-2 IMMUNOHISTOCHEMISTRY DETECTION IN EQUINE RESPIRATORY TISSUE**. Slides made from ethanol-fixed and anatomically paired formalin-fixed paraffin-embedded equine respiratory tracheal epithelium from 3 healthy abattoir horses (AH4, AH6, AH11), were stained using Muc5ac-2 purified antibody at 1:100 dilution incubated overnight and detected using DAB colour detection (without antigen retrieval step for ethanol-fixed tissue; with antigen retrieval step for formalin fixed tissue) with or without addition of equine saliva (1:100) to antibody incubation step to block keratin binding cross-reactivity. Haematoxylin counterstain. A. Ethanol-fixed tissue B. Formalin-fixed tissue. Original magnification x 40. Scale bar 100 µm. Representative images shown. Ker- = keratin blocked with addition of saliva to antibody, Ker+ = no keratin blockade. E = epithelium, SM = sub-mucosa.

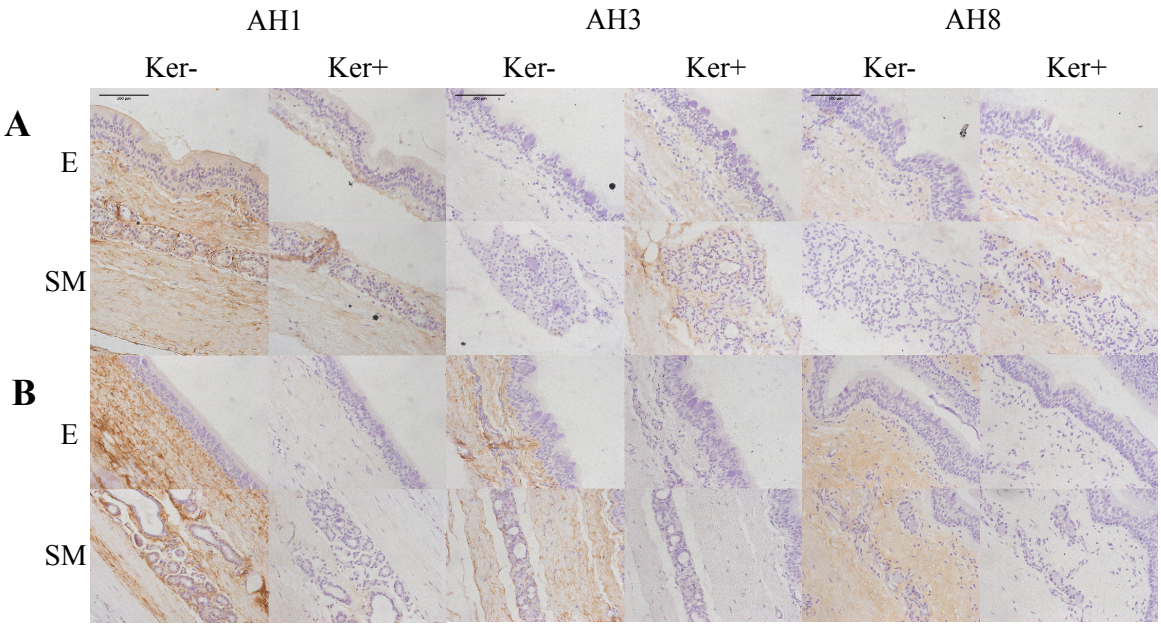

Fig. AII.5 **HISTORICAL LUNG FUNCTION DATA FROM TISSUE BANK (TB) EXPERIMENTAL HERD HORSES**. These data were previously obtained by our collaborators, Dr. J.P. Lavoie and colleagues (University of Montreal), in earlier experiments not related to the current study, to confirm a diagnosis of RAO. A diagnosis of RAO is made if maximal change in pleural pressure (ΔPpl_{max}) is increased by more than 15 cm H_2O, accompanied by a BALF neutrophil percentage of over 25% when the horse is housed in an exposed environment (Robinson, 2001). The experimental RAO horses all showed a measurable change in ΔPpl_{max} of more than 15 cm H_2O, which was also accompanied by > 25% neutrophils in BALF (data not shown) during exposure; whilst the control horses did not. **A**. Control horses Tb1 - Tb-8. **B**. RAO horses Tb-9 – Tb-19. Symbols refer to individual horses as indicated by designated numerical assignment Tb1 - Tb19 in the key.

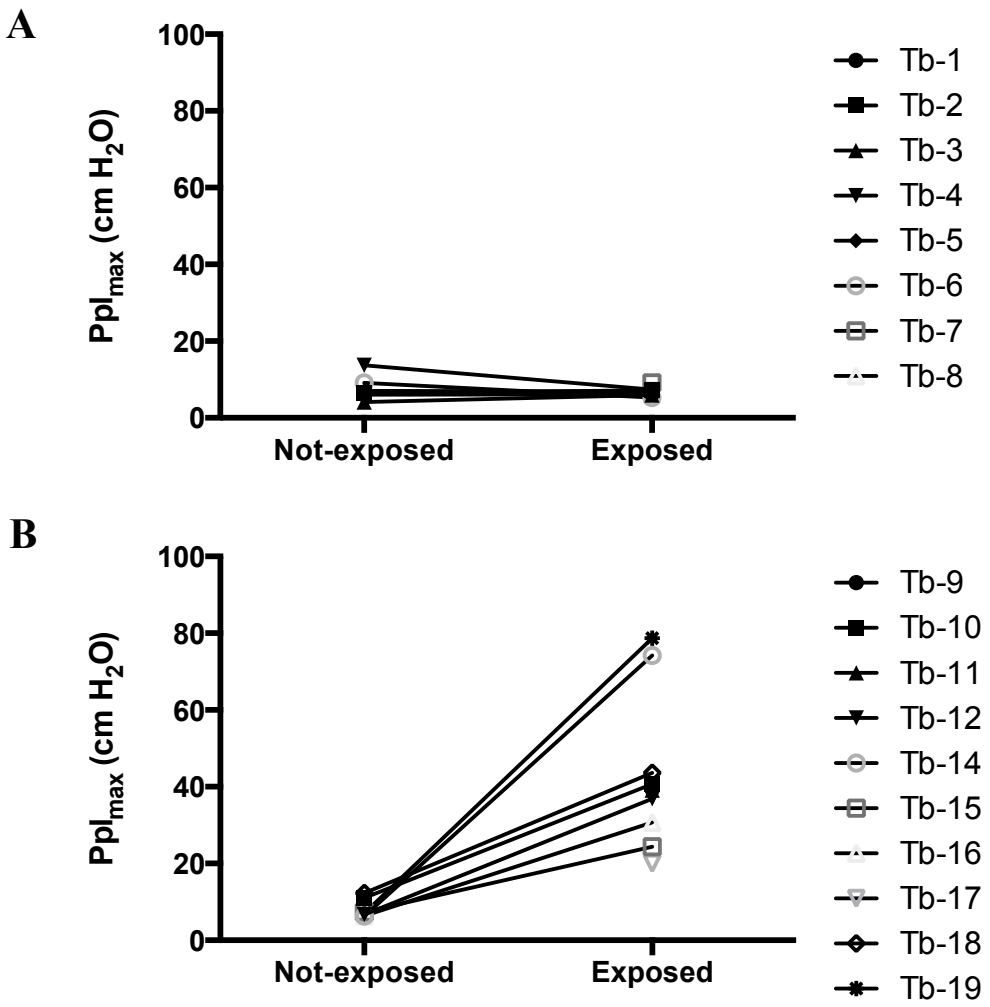

Fig. AII.6 **GROUPED CONTROL AND RAO HORSE EXPOSED AND NOT-EXPOSED SUB-GROUPS DIVIDED INTO ANATOMICAL AREAS: TRACHEA VS LUNG. DATA FROM PAS AB AND MUC5B STAINING OF RESPIRATORY TISSUES**. Tissue sections from each of 8 healthy control horses and 11 RAO-diseased horses from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections, were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Multiple t-test grouped analysis of PAS AB and Muc5b staining data for RAO and control horses divided into exposed and not-exposed subgroups (according to controlled environmental housing conditions at time of euthanasia) further subdivided into information for separate anatomical areas, trachea (T) and mid-lung bronchi (L) is shown to assess inter-stain variation in the parameters A-D. * $P < 0.05$. PAS AB light grey bars, Muc5b dark grey bars. RAO and control (Ctrl) horses were kept in either exposed (E) or not-exposed (NE) controlled environments prior to euthanasia. Graphs show mean +SEM.

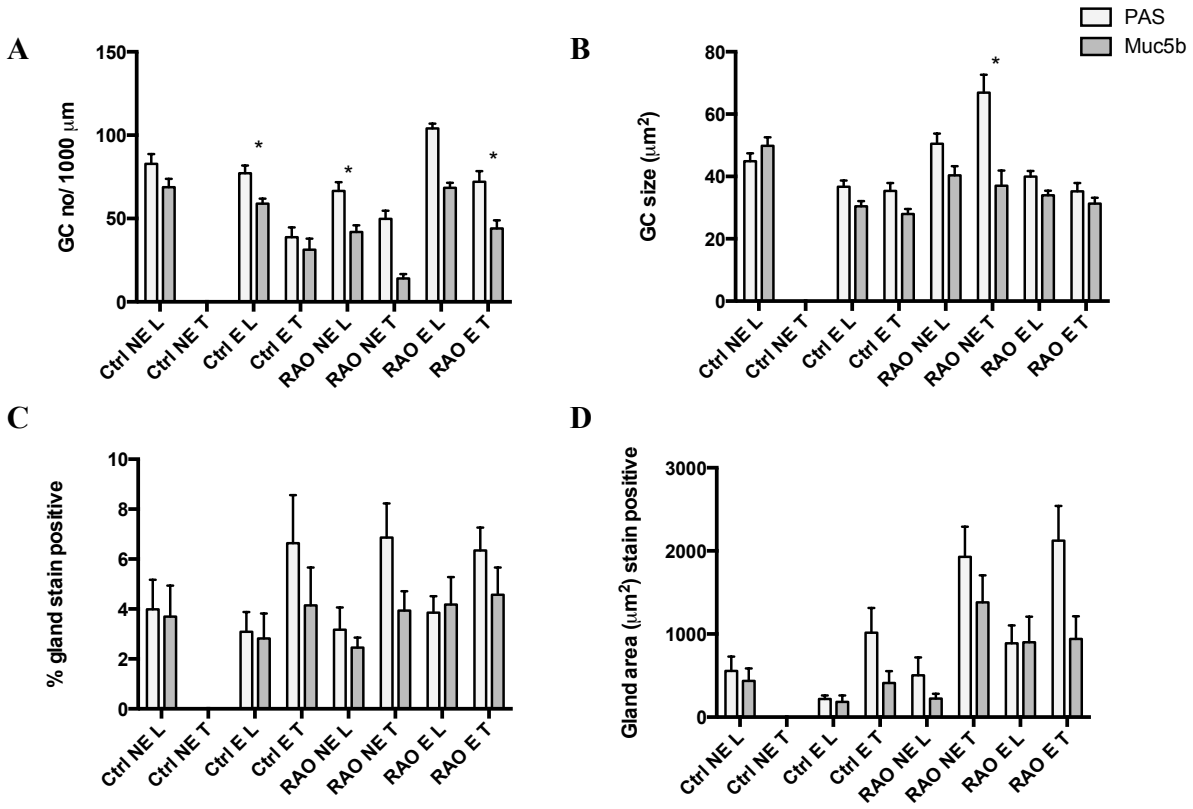

Table AII.1 **GROUPED CONTROL AND RAO HORSE EXPOSED AND NOT-EXPOSED SUB-GROUPS DIVIDED INTO ANATOMICAL AREAS: TRACHEA VS BRONCHI**. Tissue sections from each of 8 healthy control horses and 11 RAO-diseased horses from tissue bank formalin-fixed tissue from caudal trachea and mid lung sections, were cut in serial section and stained for PAS AB and Muc5b. For each slide, 1 - 9 x 20 magnification images of epithelium and sub-mucosa were analysed for mucin-cell positive staining to produce the following parameters: **A**. Number of GC per 1000 µm of epithelium. **B**. Mean GC size (μm^2). **C**. Percentage of sub-mucosal gland mucin-stain positive. **D**. Area of sub-mucosal gland mucin-stain positive (μm^2). Grouped analysis of data for RAO and control horses divided into exposed and not-exposed subgroups (according to controlled environmental housing conditions at time of euthanasia) further subdivided into information for separate anatomical areas, trachea (T) and mid-lung bronchi (L) is shown using Kruskall-Wallis ANOVA multiple group comparisons to assess inter-group variation in the parameters A-D. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. RAO and control (Ctrl) horses were kept in either exposed (E) or not-exposed (NE) controlled environments prior to euthanasia.

Staining method used	Group & sub-group comparison	Within-group sub-group comparison	A: GC number per 1000 µm epithelium	B: GC size	C: Gland % stian positive	D: Gland area stain positive
PAS AB	Control vs RAO	NE L vs NE L	*	NSD	NSD	NSD
PAS AB	Control vs RAO	NE T v NE T	n/a	n/a	n/a	n/a
PAS AB	Control vs RAO	EL vs EL	**	NSD	NSD	NSD
PAS AB	Control vs RAO	ET vs ET	*	NSD	NSD	NSD
PAS AB	Control NE vs Control E	L vs L	NSD	NSD	NSD	NSD
PAS AB	Control NE vs Control E	T vs T	n/a	n/a	n/a	n/a
PAS AB	Control NE vs Control NE	L vs T	n/a	n/a	n/a	n/a
PAS AB	Control E vs Control E	L vs T	****	NSD	NSD	NSD
PAS AB	RAO NE vs RAO E	L vs L	****	NSD	NSD	NSD
PAS AB	RAO NE vs RAO E	T vs T	*	***	NSD	NSD
PAS AB	RAO NE vs RAO NE	L vs T	NSD	NSD	NSD	**
PAS AB	RAO E vs RAO E	L vs T	****	NSD	NSD	**
Muc5b	Control vs RAO	NE L vs NE L	**	NSD	NSD	NSD
Muc5b	Control vs RAO	NE T v NE T	n/a	n/a	n/a	n/a
Muc5b	Control vs RAO	EL vs EL	NSD	NSD	NSD	NSD
Muc5b	Control vs RAO	ET vs ET	NSD	NSD	NSD	NSD
Muc5b	Control NE vs Control E	L vs L	NSD	****	NSD	NSD
Muc5b	Control NE vs Control E	T vs T	n/a	n/a	n/a	n/a
Muc5b	Control NE vs Control NE	L vs T	n/a	n/a	n/a	n/a
Muc5b	Control E vs Control E	L vs T	*	NSD	NSD	NSD
Muc5b	RAO NE vs RAO E	L vs L	****	NSD	NSD	NSD
Muc5b	RAO NE vs RAO E	T vs T	NSD	NSD	NSD	NSD
Muc5b	RAO NE vs RAO NE	L vs T	NSD	NSD	NSD	*
Muc5b	RAO E vs RAO E	L vs T	**	NSD	NSD	NSD

REFERENCES

Abraham, G., Zizzadoro, C., Kacza, J., Ellenberger, C., Abs, V., Franke, J., Schoon, H.-A., Seeger, J., Tesfaigzi, Y. and Ungemach, F. (2011) Growth and differentiation of primary and passaged equine bronchial epithelial cells under conventional and air-liquid-interface culture conditions. *BMC Veterinary Research* **7**, 1-13.

Ainsworth, D.M., Grunig, G., Matychak, M.B., Young, J., Wagner, B., Erb, H.N. and Antczak, D.F. (2003) Recurrent airway obstruction (rao) in horses is characterized by ifn-gamma and il-8 production in bronchoalveolar lavage cells. *Vet Immunol Immunopathol* **96**, 83-91.

Ainsworth, D.M., Wagner, B., Erb, H.N., Young, J.C. and Retallick, D.E. (2007) Effects of in vitro exposure to hay dust on expression of interleukin-17, -23, -8, and -1beta and chemokine (c-x-c motif) ligand 2 by pulmonary mononuclear cells isolated from horses chronically affected with recurrent airway disease. *Am J Vet Res* **68**, 1361-1369.

Ainsworth, D.M., Wagner, B., Franchini, M., Grunig, G., Erb, H.N. and Tan, J.Y. (2006) Time-dependent alterations in gene expression of interleukin-8 in the bronchial epithelium of horses with recurrent airway obstruction. *Am J Vet Res* **67**, 669-677.

Allen, K. and Franklin, S. (2007) Rao and iad: Respiratory disease in horses revisited. *In Practice* **29**, 76 - 82.

Art, T., Kirschvink, N., Smith, N., Votion, D. and Lekeux, P. (1999) Cardiorespiratory measurements and the indices of oxidative stress in exercising copd horses. *Equine Vet J* **Supplement: Equine Exercise Physiology 5**, 83 - 87.

Bartner, L.R., Robinson, N.E., Kiupel, M. and Tesfaigzi, Y. (2006) Persistent mucus accumulation: A consequence of delayed bronchial mucous cell apoptosis in rao-affected horses? *American journal of physiology. Lung cellular and molecular physiology* **291**, L602-609.

Boden, L.A., Parkin, T.D., Yates, J., Mellor, D. and Kao, R.R. (2012) Summary of current knowledge of the size and spatial distribution of the horse population within great britain. *BMC Vet Res* **8**, 43.

Bowles, K.S., Beadle, R.E., Mouch, S., Pourciau, S.S., Littlefield-Chabaud, M.A., Le Blanc, C., Mistric, L., Fermaglich, D. and Horohov, D.W. (2002) A novel model for equine recurrent airway obstruction. *Vet Immunol Immunopathol* **87**, 385-389.

Buisine, M.P., Desseyn, J.L., Porchet, N., Degand, P., Laine, A. and Aubert, J.P. (1998) Genomic organization of the 3'-region of the human muc5ac mucin gene: Additional evidence for a common ancestral gene for the 11p15.5 mucin gene family. *Biochem J* **332 (Pt 3)**, 729-738.

Buisine, M.P., Devisme, L., Copin, M.C., Durand-Reville, M., Gosselin, B., Aubert, J.P. and Porchet, N. (1999) Developmental mucin gene expression in the human respiratory tract. *Am J Respir Cell Mol Biol* **20**, 209-218.

Burgel, P.-R.g., Montani, D., Danel, C., Dusser, D.J. and Nadel, J.A. (2007) A morphometric study of mucins and small airway plugging in cystic fibrosis. *Thorax* **62**, 153-161.

Campbell, A.M., Chanez, P., Vignola, A.M., Bousquet, J., Couret, I., Michel, F.B. and Godard, P. (1993) Functional characteristics of bronchial epithelium obtained by brushing from asthmatic and normal subjects. *Am Rev Respir Dis* **147**, 529-534.

Carlstedt, I., Lindgren, H. and Sheehan, J.K. (1983a) The macromolecular structure of human cervical-mucus glycoproteins. Studies on fragments obtained after reduction of disulphide bridges and after subsequent trypsin digestion. *Biochem J* **213**, 427-435.

Carlstedt, I., Lindgren, H., Sheehan, J.K., Ulmsten, U. and Wingerup, L. (1983b) Isolation and characterization of human cervical-mucus glycoproteins. *Biochem. J.* **211**, 13-22.

Carlstedt, I., Sheehan, J.K., Corfield, A.P. and Gallagher, J.T. (1985) Mucous glycoproteins: A gel of a problem. *Essays in biochemistry* **20**, 40-76.

Chen, Y., Zhao, Y.H., Kalaslavadi, T.B., Hamati, E., Nehrke, K., Le, A.D., Ann, D.K. and Wu, R. (2004) Genome-wide search and identification of a novel gel-forming mucin muc19/muc19 in glandular tissues. *Am J Respir Cell Mol Biol* **30**, 155-165.

Christley, R.M., Hodgson, D.R., Rose, R.J., Hodgson, J.L., Wood, J.L.N. and Reid, S.W.J. (2001) Coughing in thoroughbred racehorses: Risk factors and tracheal endoscopic and cytological findings. *Vet Rec.* **148**, 99-104.

Clamp, J.R., Allen, A., Gibbons, R.A. and Roberts, G.P. (1978) Chemical aspects of mucus. *Br Med Bull* **34**, 25-41.

Coombs, S.L. and Webbon, P.M. (1987) Observations on tracheal mucociliary clearance in horses. *Tierarztl Prax Suppl* **2**, 5-9.

Cordeau, M.-E., Joubert, P., Dewachi, O., Hamid, Q. and Lavoie, J.-P. (2004) Il-4, il-5 and ifn-[gamma] mrna expression in pulmonary lymphocytes in equine heaves. *Veterinary Immunology and Immunopathology* **97**, 87-96.

Couetil, L.L., Rosenthal, F.S., DeNicola, D.B. and Chilcoat, C.D. (2001) Clinical signs, evaluation of bronchoalveolar lavage fluid, and assessment of pulmonary function in horses with inflammatory respiratory disease. *American Journal of Veterinary Research* **62**, 538-546.

Creeth, J.M. (1978) Constituents of mucus and their separation. *Br. Med. Bull.* **34**, 17-24.

Dabbagh, K., Takeyama, K., Lee, H.M., Ueki, I.F., Lausier, J.A. and Nadel, J.A. (1999) Il-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J. Immunol.* **162**, 6233-6237.

Davies, J.R., Hovenberg, H.W., Linden, C.J., Howard, R., Richardson, P.S., Sheehan, J.K. and Carlstedt, I. (1996) Mucins in airway secretions from healthy and chronic bronchitic subjects. *Biochem. J.* **313**, 431-439.

Davis, E. and Rush, B.R. (2002) Equine recurrent airway obstruction: Pathogenesis, diagnosis, and patient management. *Vet. Clin. N. Am.-Equine Pract.* **18**, 453-467.

Debrue, M., Hamilton, E., Joubert, P., Lajoie-Kadoch, S. and Lavoie, J.P. (2005) Chronic exacerbation of equine heaves is associated with an increased expression of interleukin-17 mrna in bronchoalveolar lavage cells. *Vet Immunol Immunopathol* **105**, 25-31.

Derksen, F.J., Robinson, N.E., Scott, J.S. and Stick, J.A. (1988) Aerosolized micropolyspora faeni antigen as a cause of pulmonary dysfunction in ponies with recurrent airway obstruction (heaves). *Am J Vet Res* **49**, 933-938.

Desseyn, J.L., Aubert, J.P., Van Seuningen, I., Porchet, N. and Laine, A. (1997a) Genomic organization of the 3' region of the human mucin gene muc5b. *J Biol Chem* **272**, 16873-16883.

Desseyn, J.L., Buisine, M.P., Porchet, N., Aubert, J.P. and Laine, A. (1998) Genomic organization of the human mucin gene muc5b. Cdna and genomic sequences upstream of the large central exon. *J Biol Chem* **273**, 30157-30164.

Desseyn, J.L., Guyonnet-Duperat, V., Porchet, N., Aubert, J.P. and Laine, A. (1997b) Human mucin gene muc5b, the 10.7-kb large central exon encodes various alternate subdomains resulting in a super-repeat. Structural evidence for a 11p15.5 gene family. *J Biol Chem* **272**, 3168-3178.

Dixon, P.M., McGorum, B.C. and Pirie, R.S. (2002) Inflammatory airway disease: European clinician's perspective. In: *Inflammatory Airway Disease: Defining the Syndrome*, Eds: A. Hoffman, N.E. Robinson and J.F. Wade, R. & W. Publications (Newmarket) Ltd., Boston, U.S.A. pp 7 - 8.

Dixon, P.M., Railton, D.I. and McGorum, B.C. (1995a) Equine pulmonary disease: A case control study of 300 referred cases. Part 1: Examination techniques, diagnostic criteria and diagnoses. *Equine Vet J* **27**, 416-421.

Dixon, P.M., Railton, D.I. and McGorum, B.C. (1995b) Equine pulmonary disease: A case control study of 300 referred cases. Part 2: Details of animals and of historical and clinical findings. *Equine Vet J* **27**, 422-427.

Douwes, J., Gibson, P., Pekkanen, J. and Pearce, N. (2002) Non-eosinophilic asthma: Importance and possible mechanisms. *Thorax* **57**, 643-648.

Durham, A. (2001) Update on therapeutics for obstructive pulmonary diseases in horses. *In Practice* **23**, 474 - 481.

Escande, F., Aubert, J.P., Porchet, N. and Buisine, M.P. (2001) Human mucin gene muc5ac: Organization of its 5'-region and central repetitive region. *Biochem J* **358**, 763-772.

Fahy, J.V. (2002) Goblet cell and mucin gene abnormalities in asthma. *Chest* **122**, 320S-326S.

Fairbairn, S.M., Page, C.P., Lees, P. and Cunningham, F.M. (1993) Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen exposure. *Clinical & Experimental Allergy* **23**, 821-828.

Felgentreff, K., Beisswenger, C., Griese, M., Gulder, T., Bringmann, G. and Bals, R. (2006) The antimicrobial peptide cathelicidin interacts with airway mucus. *Peptides* **27**, 3100-3106.

Frellstedt, L., Gosset, P., Kervoaze, G., Desmet, C., Pirottin, D., Bureau, F., Lekeux, P. and Art, T. (2013) A method for the culture of equine bronchial epithelial cells derived from bronchoscopy in live horses. In: *World Equine Airway Symposium*, Calgary, Canada. p 46.

Fulcher, M.L., Gabriel, S., Burns, K.A., Yankaskas, J.R. and Randell, S.H. (2005) Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* **107**, 183-206.

Gerber, V., Baleri, D., Klukowska-Rotzler, J., Swinburne, J.E. and Dolf, G. (2009a) Mixed inheritance of equine recurrent airway obstruction. *J Vet Intern Med* **23**, 626-630.

Gerber, V., De Feijter-Rupp, H., Wagner, J., Venta, P., Harkema, J.R. and Robinson, N.E. (2009b) Differential association of muc5ac and clca1 expression in small cartilaginous airways of rao-affected and control horses. *Equine Vet J* **41**, 817-823.

Gerber, V., Gehr, P., Straub, R., Frenz, M., King, M. and Im Hof, V. (1997) Mucus quality on horse tracheal epithelium: Microscopic grading based on transparency. *Respir Physiol* **107**, 67-74.

Gerber, V., Jefcoat, A.M., Hotchkiss, J.A., King, M. and Robinson, N.E. (2002) Quantifying and characterising mucus in the airways. In: *Inflammatory Airway Disease: Defining the Syndrome*, Eds: A. Hoffman, N.E. Robinson and J.F. Wade, R. & W. Publications (Newmarket) Ltd., Boston, U.S.A. pp 59 - 61.

Gerber, V., King, M., Schneider, D.A. and Robinson, N.E. (2000) Tracheobronchial mucus viscoelasticity during environmental challenge in horses with recurrent airway obstruction. *Equine Vet. J.* **32**, 411-417.

Gerber, V. and Robinson, N.E. (2007) Airway secretions and mucociliary function. In: *Equine respiratory medicine and surgery*, 1 edn., Eds: B.C. McGorum, P.M. Dixon, N.E. Robinson and J. Schumacher, Saunders Elsevier. pp 55 - 69.

Gerber, V., Robinson, N.E., Venta, P.J., Rawson, J., Jefcoat, A.M. and Hotchkiss, J.A. (2003) Mucin genes in horse airways: Muc5ac, but not muc2, may play a role in recurrent airway obstruction. *Equine Vet. J.* **35**, 252-257.

Gerber, V., Straub, R., Marti, E., Hauptman, J., Herholz, C., King, M., Imhof, A., Tahon, L. and Robinson, N.E. (2004) Endoscopic scoring of mucus quantity and quality: Observer and horse variance and relationship to inflammation, mucus viscoelasticity and volume. *Equine Vet. J.* **36**, 576-582.

Giguère, S., Viel, L., Lee, E., MacKay, R.J., Hernandez, J. and Franchini, M. (2002) Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Veterinary Immunology and Immunopathology* **85**, 147-158.

Groneberg, D.A., Eynott, P.R., Lim, S., Oates, T., Wu, R., Carlstedt, I., Roberts, P., McCann, B., Nicholson, A.G., Harrison, B.D. and Chung, K.F. (2002a) Expression of respiratory mucins in fatal status asthmaticus and mild asthma. *Histopathology* **40**, 367-373.

Groneberg, D.A., Eynott, P.R., Oates, T., Lim, S., Wu, R., Carlstedt, I., Nicholson, A.G. and Chung, K.F. (2002b) Expression of muc5ac and muc5b mucins in normal and cystic fibrosis lung. *Respiratory Medicine* **96**, 81-86.

Haldar, P. and Pavord, I.D. (2007) Noneosinophilic asthma: A distinct clinical and pathologic phenotype. *Journal of Allergy and Clinical Immunology* **119**, 1043-1052.

Hasnain, S.Z., Evans, C.M., Roy, M., Gallagher, A.L., Kindrachuk, K.N., Barron, L., Dickey, B.F., Wilson, M.S., Wynn, T.A., Grencis, R.K. and Thornton, D.J. (2011) Muc5ac: A critical component mediating the rejection of enteric nematodes. *The Journal of experimental medicine* **208**, 893-900.

Hattrup, C.L. and Gendler, S.J. (2008) Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol* **70**, 431-457.

Herszberg, B., Ramos-Barbon, D., Tamaoka, M., Martin, J.G. and Lavoie, J.P. (2006) Heaves, an asthma-like equine disease, involves airway smooth muscle remodeling. *J Allergy Clin Immunol* **118**, 382-388.

Holloway, Beghé and Holgate (1999) The genetic basis of atopic asthma. *Clinical & Experimental Allergy* **29**, 1023-1032.

Hotchkiss, J.W., Reid, S.W. and Christley, R.M. (2007) A survey of horse owners in great britain regarding horses in their care. Part 2: Risk factors for recurrent airway obstruction. *Equine Vet J* **39**, 301-308.

Hovenberg, H.W., Davies, J.R. and Carlstedt, I. (1996a) Different mucins are produced by the surface epithelium and the submucosa in human trachea: Identification of muc5ac as a major mucin from the goblet cells. *Biochem. J.* **318**, 319-324.

Hovenberg, H.W., Davies, J.R., Herrmann, A., Lindén, C.-J. and Carlstedt, I. (1996b) Muc5ac, but not muc2, is a prominent mucin in respiratory secretions. *Glycoconjugate Journal* **13**, 839-847.

Husain, A.N. and Kumar, V. (2005) The lung: Asthma. In: *Robbins and cotran pathologic basis of disease*, 7 edn., Eds: V. Kumar, A.K. Abbas and F. N., Elsevier Saunders, Philadelphia, U.S.A. pp 723 - 727.

Jefcoat, A.M., Hotchkiss, J.A., Gerber, V., Harkema, J.R., Basbaum, C.B. and Robinson, N.E. (2001) Persistent mucin glycoprotein alterations in equine recurrent airway obstruction. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **281**, L704-L712.

Jeffery, P. and Li, D. (1997) Airway mucosa: Secretory cells, mucus and mucin genes. *European Respiratory Journal* **10**, 1655-1662.

Jost, U., Klukowska-Rotzler, J., Dolf, G., Swinburne, J.E., Ramseyer, A., Bugno, M., Burger, D., Blott, S. and Gerber, V. (2007) A region on equine chromosome 13 is linked to recurrent airway obstruction in horses. *Equine Vet J* **39**, 236-241.

Kaup, F.J., Drommer, W., Damsch, S. and Deegen, E. (1990) Ultrastructural findings in horses with chronic obstructive pulmonary disease (copd). Ii: Pathomorphological changes of the terminal airways and the alveolar region. *Equine Vet J* **22**, 349-355.

Kelsen, S.G., Mardini, I.A., Zhou, S., Benovic, J.L. and Higgins, N.C. (1992) A technique to harvest viable tracheobronchial epithelial cells from living human donors. *Am. J. Respir. Cell Mol. Biol.* **7**, 66-72.

Kesimer, M., Scull, M., Brighton, B., DeMaria, G., Burns, K., O'Neal, W., Pickles, R.J. and Sheehan, J.K. (2009) Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: A possible role in innate defense. *The FASEB Journal* **23**, 1858-1868.

King, M., Gilboa, A., Meyer, F.A. and Silberberg, A. (1974) On the transport of mucus and its rheologic simulants in ciliated systems. *Am Rev Respir Dis* **110**, 740-745.

Kirkham, S., Sheehan, J.K., Knight, D., Richardson, P.S. and Thornton, D.J. (2002) Heterogeneity of airways mucus: Variations in the amounts and glycoforms of the major oligomeric mucins muc5ac and muc5b. *Biochem. J.* **361**, 537-546.

Knowles, M.R. and Boucher, R.C. (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *The Journal of Clinical Investigation* **109**, 571-577.

Lamblin, G., Lhermitte, M., Degand, P., Roussel, P. and Slayter, H.S. (1979) Chemical and physical properties of human bronchial mucus glycoproteins. *Biochimie* **61**, 23-43.

Larson, V.L. and Busch, R.H. (1985) Equine tracheobronchial lavage: Comparison of lavage cytologic and pulmonary histopathologic findings. *Am J Vet Res* **46**, 144-146.

Lavoie, J.P. (2007) Recurrent airway obstruction (heaves) and summer-pasture-associated obstructive pulmonary disease. In: *Equine respiratory medicine and surgery*, Eds: B.C. McGorum, P.M. Dixon, N.E. Robinson and S. J., Elsevier Limited. pp 565 - 589.

Leclere, M., Lavoie-Lamoureux, A., Gelinas-Lymburner, E., David, F., Martin, J.G. and Lavoie, J.P. (2011a) Effect of antigenic exposure on airway smooth muscle remodeling in an equine model of chronic asthma. *Am J Respir Cell Mol Biol* **45**, 181-187.

Leclere, M., Lavoie-Lamoureux, A., Joubert, P., Relave, F., Setlakwe, E.L., Beauchamp, G., Couture, C., Martin, J.G. and Lavoie, J.P. (2012) Corticosteroids and antigen avoidance decrease airway smooth muscle mass in an equine asthma model. *Am J Respir Cell Mol Biol* **47**, 589-596.

Leclere, M., Lavoie-Lamoureux, A. and Lavoie, J.-P. (2011b) Heaves, an asthma-like disease of horses. *Respirology* **16**, 1027-1046.

Leclere, M., Lefebvre-Lavoie, J., Beauchamp, G. and Lavoie, J.-P. (2010) Efficacy of oral prednisolone and dexamethasone in horses with recurrent airway obstruction in the presence of continuous antigen exposure. *Equine Vet. J.* **42**, 316-321.

Léguillette, R. (2003) Recurrent airway obstruction - heaves. *Veterinary Clinics of North America: Equine Practice* **19**, 63-86.

Longphre, M., Li, D., Gallup, M., Drori, E., OrdoÃ±ez, C.L., Redman, T., Wenzel, S., Bice, D.E., Fahy, J.V. and Basbaum, C. (1999) Allergen-induced il-9 directly stimulates mucin transcription in respiratory epithelial cells. *The Journal of Clinical Investigation* **104**, 1375-1382.

Louahed, J., Toda, M., Jen, J., Hamid, Q., Renauld, J.C., Levitt, R.C. and Nicolaides, N.C. (2000) Interleukin-9 upregulates mucus expression in the airways. *Am. J. Respir. Cell Mol. Biol.* **22**, 649-656.

Lugo, J., Harkema, J.R., deFeijter-Rupp, H., Bartner, L., Boruta, D. and Robinson, N.E. (2006) Airway inflammation is associated with mucous cell metaplasia and increased intraepithelial stored mucosubstances in horses. *Veterinary journal* **172**, 293-301.

Mair, T.S. (1996) Obstructive pulmonary disease in 18 horses at summer pasture. *Vet Rec* **138**, 89-91.

Majima, Y., Harada, T., Shimizu, T., Takeuchi, K., Sakakura, Y., Yasuoka, S. and Yoshinaga, S. (1999) Effect of biochemical components on rheologic properties of nasal mucus in chronic sinusitis. *Am. J. Respir. Crit. Care Med.* **160**, 421-426.

Marti, E., Gerber, H., Essich, G., Oulehla, J. and Lazary, S. (1991) The genetic basis of equine allergic diseases. 1. Chronic hypersensitivity bronchitis. *Equine Vet J* **23**, 457-460.

Matsui, H., Randell, S.H., Peretti, S.W., Davis, C.W. and Boucher, R.C. (1998) Coordinated clearance of periciliary liquid and mucus from airway surfaces. *The Journal of Clinical Investigation* **102**, 1125-1131.

Matthews, A.G., Hackett, I.J. and Lawton, W.A. (1988) The mucolytic effect of sputolosin in horses with respiratory disease. *Vet Rec* **122**, 106-108.

Mazan, M.R., Deveney, E.F., DeWitt, S., Bedenice, D. and Hoffman, A. (2004) Energetic cost of breathing, body composition, and pulmonary function in horses with recurrent airway obstruction. *J Appl Physiol* **97**, 91-97.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E. (1993a) Responses of horses affected with chronic obstructive pulmonary disease to inhalation challenges with mould antigens. *Equine Vet J* **25**, 261-267.

McGorum, B.C., Dixon, P.M. and Halliwell, R.E.W. (1993b) Phenotypic analysis of peripheral blood and bronchoalveolar lavage fluid lymphocytes in control and chronic obstructive pulmonary disease affected horses, before and after natural (hay and straw) challenges'. *Veterinary Immunology and Immunopathology* **36**, 207-222.

McPherson, E.A., Lawson, G.H., Murphy, J.R., Nicholson, J.M., Fraser, J.A., Breeze, R.G. and Pirie, H.M. (1978) Chronic obstructive pulmonary disease (copd): Identification of affected horses. *Equine Vet J* **10**, 47-53.

McPherson, E.A., Lawson, G.H.K., Murphy, J.R., Nicholson, J.M., Breeze, R.G. and Pirie, H.M. (1979) Chronic obstructive pulmonary disease (copd): Factors influencing the occurrence. *Equine Vet. J.* **11**, 167-171.

Meyer, F.A. (1976) Mucus structure: Relation to biological transport function. *Biorheology* **13**, 49-58.

Naylor, J.M., Clark, E.G. and Clayton, H.M. (1992) Chronic obstructive pulmonary disease: Usefulness of clinical signs, bronchoalveolar lavage, and lung biopsy as diagnostic and prognostic aids. *Can Vet J* **33**, 591-598.

Nyman, G., Lindberg, R., Weckner, D., BjÖRk, M., Kvart, C., Persson, S.G.B., Gustafsson, H. and Hedenstierna, G. (1991) Pulmonary gas exchange correlated to clinical signs and lung pathology in horses with chronic bronchiolitis. *Equine Vet. J.* **23**, 253-260.

Ordonez, C.L., Khashayar, R., Wong, H.H., Ferrando, R.O.N., Wu, R., Hyde, D.M., Hotchkiss, J.A., Zhang, Y., Novikov, A., Dolganov, G. and Fahy, J.V. (2001) Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am. J. Respir. Crit. Care Med.* **163**, 517-523.

Oslund, K.L., Adamson, G. and Wu, R. (2010) Evaluation of muc5ac expression and upregulation in airway epithelial cells of horses. *Am J Vet Res* **71**, 690-696.

Perez-Vilar, J. and Hill, R.L. (1999) The structure and assembly of secreted mucins. *J. Biol. Chem.* **274**, 31751-31754.

Pigny, P., Guyonnet-Duperat, V., Hill, A.S., Pratt, W.S., Galiegue-Zouitina, S., d'Hooge, M.C., Laine, A., Van-Seuningen, I., Degand, P., Gum, J.R., Kim, Y.S., Swallow, D.M., Aubert, J.-P. and Porchet, N. (1996) Human mucin genes assigned to 11p15.5: Identification and organization of a cluster of genes. *Genomics* **38**, 340-352.

Pirie, M., Pirie, H.M., Cranston, S. and Wright, N.G. (1990a) An ultrastructural study of the equine lower respiratory tract. *Equine Vet J* **22**, 338-342.

Pirie, M., Pirie, H.M. and Wright, N.G. (1990b) A scanning electron microscopic study of the equine upper respiratory tract. *Equine Vet J* **22**, 333-337.

Pirie, R.S., Dixon, P.M., Collie, D.D.S. and McGorum, B.C. (2001) Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. *Equine Vet. J.* **33**, 311-318.

Quintana, A.M., Landolt, G.A., Annis, K.M. and Hussey, G.S. (2011) Immunological characterization of the equine airway epithelium and of a primary equine airway epithelial cell culture model. *Vet Immunol Immunopathol* **140**, 226-236.

Racine, J., Gerber, V., Feutz, M.M., Riley, C.P., Adamec, J., Swinburne, J.E. and Couetil, L.L. (2011) Comparison of genomic and proteomic data in recurrent airway obstruction affected horses using ingenuity pathway analysis(r). *BMC Vet Res* **7**, 48.

Racklyeft, D.J. and Love, D.N. (1990) Influence of head posture on the respiratory tract of healthy horses. *Australian Veterinary Journal* **67**, 402-405.

Raidal, S.L., Love, D.N. and Bailey, G.D. (1996) Effects of posture and accumulated airway secretions on tracheal mucociliary transport in the horse. *Aust Vet J* **73**, 45-49.

Ramseyer, A., Gaillard, C., Burger, D., Straub, R., Jost, U., Boog, C., Marti, E. and Gerber, V. (2007) Effects of genetic and environmental factors on chronic lower airway disease in horses. *J Vet Intern Med* **21**, 149-156.

Randell, S.H. and Boucher, R.C. (2006) Effective mucus clearance is essential for respiratory health. *Am. J. Respir. Cell Mol. Biol.* **35**, 20-28.

Raynal, B.D., Hardingham, T.E., Sheehan, J.K. and Thornton, D.J. (2003) Calcium-dependent protein interactions in muc5b provide reversible cross-links in salivary mucus. *J Biol Chem* **278**, 28703-28710.

Reid, L. and Clamp, J.R. (1978) The biochemical and histochemical nomenclature of mucus. *Br Med Bull* **34**, 5-8.

Robin, C.A., Lo Iacono, G., Gubbins, S., Wood, J.L. and Newton, J.R. (2013) The accuracy of the national equine database in relation to vector-borne disease risk modelling of horses in great britain. *Equine Vet J* **45**, 302-308.

Robinson, N.E. (2001) International workshop on equine chronic airway disease. Michigan state university 16-18 june 2000. *Equine Vet J* **33**, 5-19.

Robinson, N.E., Berney, C., DeFeijter-Rupp, H., Jefcoat, A.M., Cornelisse, C., Gerber, V. and Derksen, F.J. (2002) Mucus, cough, airway obstruction and inflammation. In: *Inflammatory Airway Disease: Defining the Syndrome*, Ed: A. Hoffman, Robinson, N.E., and Wade, J.F., R. & W. Publications (Newmarket) Ltd., Boston, U.S.A. pp 13 - 15.

Robinson, N.E., Berney, C., Eberhart, S., deFeijter-Rupp, H.L., Jefcoat, A.M., Cornelisse, C.J., Gerber, V.M. and Derksen, F.J. (2003) Coughing, mucus accumulation, airway obstruction, and airway inflammation in control horses and horses affected with recurrent airway obstruction. *American Journal of Veterinary Research* **64**, 550-557.

Robinson, N.E., Derksen, F.J., Jackson, C.A., Peroni, D. and Gerber, V. (2001) Management of heaves. *Equine Veterinary Education* **13**, 247-259.

Robinson, N.E., Derksen, F.J., Olszewski, M.A. and Buechner-Maxwell, V.A. (1996) The pathogenesis of chronic obstructive pulmonary disease of horses. *British Veterinary Journal* **152**, 283-306.

Robinson, N.E. and Furlow, P.W. (2007) Anatomy of the respiratory system. In: *Equine respiratory medicine and surgery*, 1 edn., Eds: B.C. McGorum, P.M. Dixon, N.E. Robinson and J. Schumacher, Saunders Elsevier pp 3-17

Rogan, M., Geraghty, P., Greene, C., O'Neill, S., Taggart, C. and McElvaney, N. (2006) Antimicrobial proteins and polypeptides in pulmonary innate defence. *Respiratory Research* **7**, 29.

Romagnoli, M., Vachier, I., Vignola, A.M., Godard, P., Bousquet, J. and Chanez, P. (1999) Safety and cellular assessment of bronchial brushing in airway diseases. *Respiratory Medicine* **93**, 461-466.

Rose, M.C. and Voynow, J.A. (2006) Respiratory tract mucin genes and mucin glycoproteins in health and disease. pp 245-278.

Rousseau, K., Byrne, C., Kim, Y.S., Gum, J.R., Swallow, D.M. and Toribara, N.W. (2004) The complete genomic organization of the human muc6 and muc2 mucin genes. *Genomics* **83**, 936-939.

Rousseau, K., Cardwell, J., Newton, R., Clegg, P. and Thornton, D.J. (2011a) Increase of muc5ac and muc5b glycoprotein in equine airway mucus accumulation and characterisation of muc5ac and muc5b from primary equine airway epithelial cells in culture. In: *British Equine Veterianry Association Congress*, EVJ, Liverpool. p 83.

Rousseau, K., Cardwell, J.M., Humphrey, E., Newton, R., Knight, D., Clegg, P. and Thornton, D.J. (2011b) Muc5b is the major polymeric mucin in mucus from thoroughbred horses with and without airway mucus accumulation. *PLoS ONE* **6**, e19678.

Rousseau, K., Kirkham, S., McKane, S., Newton, R., Clegg, P. and Thornton, D.J. (2007) Muc5b and muc5ac are the major oligomeric mucins in equine airway mucus. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **292**, L1396-L1404.

Ryhner, T., Mueller, N., Balmer, V. and Gerber, V. (2008) Increased mucus accumulation in horses chronically affected with recurrent airway obstruction is not associated with up-regulation of clca1, egfr, muc5ac, bcl-2, il-13 and inf-gamma expression. *Veterinary Immunology and Immunopathology* **125**, 8-17.

Scharfman, A., Degroote, S., Beau, J., Lamblin, G., Roussel, P. and Mazurier, J. (1999) Pseudomonas aeruginosa binds to neoglycoconjugates bearing mucin carbohydrate determinants and predominantly to sialyl-lewis x conjugates. *Glycobiology* **9**, 757-764.

Schwab, U., Caldwell, S., Matychak, M.-B. and Felippe, J. (2013) A 3-d airway epithelial cell and macrophage co-culture system to study rhodococcus equi infection. *Veterinary Immunology and Immunopathology* **154**, 54-61.

Schwab, U.E., Fulcher, M.L., Randell, S.H., Flaminio, M.J. and Russell, D.G. (2010) Equine bronchial epithelial cells differentiate into ciliated and mucus producing cells in vitro. *In Vitro Cell Dev Biol Anim* **46**, 102-106.

Seahorn, T.L. and Beadle, R.E. (1993) Summer pasture-associated obstructive pulmonary disease in horses: 21 cases (1983-1991). *J Am Vet Med Assoc* **202**, 779-782.

Sheehan, J.K., Brazeau, C., Kutay, S., Pigeon, H., Kirkham, S., Howard, M. and Thornton, D.J. (2000) Physical characterization of the muc5ac mucin: A highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions. *Biochem J* **347 Pt 1**, 37-44.

Sheehan, J.K. and Carlstedt, I. (1984a) The effect of guanidinium chloride on the behaviour of human cervical-mucus glycoproteins. Evidence for unfolding regions of ordered structure in 6m-guanidinium chloride. *Biochem J* **221**, 499-504.

Sheehan, J.K. and Carlstedt, I. (1984b) Hydrodynamic properties of human cervical-mucus glycoproteins in 6m-guanidinium chloride. *Biochem. J.* **217**, 93-101.

Sheehan, J.K., Howard, M., Richardson, P.S., Longwill, T. and Thornton, D.J. (1999) Physical characterization of a low-charge glycoform of the muc5b mucin comprising the gel-phase of an asthmatic respiratory mucous plug. *Biochem. J.* **338**, 507-513.

Sheehan, J.K., Kesimer, M. and Pickles, R. (2006) Innate immunity and mucus structure and function. *Novartis Found Symp* **279**, 155-166; discussion 167-159, 216-159.

Sheehan, J.K., Kirkham, S., Howard, M., Woodman, P., Kutay, S., Brazeau, C., Buckley, J. and Thornton, D.J. (2004) Identification of molecular intermediates in the assembly pathway of the muc5ac mucin. *J. Biol. Chem.* **279**, 15698-15705.

Sheehan, J.K., Richardson, P.S., Fung, D.C., Howard, M. and Thornton, D.J. (1995) Analysis of respiratory mucus glycoproteins in asthma: A detailed study from a patient who died in status asthmaticus. *Am J Respir Cell Mol Biol* **13**, 748-756.

Shibeshi, W., Abraham, G., Kneuer, C., Ellenberger, C., Seeger, J., Schoon, H.A. and Ungemach, F.R. (2008) Isolation and culture of primary equine tracheal epithelial cells. *In Vitro Cell Dev Biol Anim* **44**, 179-184.

Sime, A., McKellar, Q. and Nolan, A. (1997) Method for the growth of equine airway epithelial cells in culture. *Res Vet Sci* **62**, 30-33.

Smith, K.C., Newton, J.R., Gower, S.M., Cade, S.M., Marlin, D.J., Deaton, C.M. and Wood, J.L.N. (2002) Cytology of inflammatory airway disease. In: *Inflammatory Airway Disease: Defining the Syndrome*, Eds: A. Hoffman, N.E. Robinson and J.F. Wade, R. & W. Publications (Newmarket) Ltd., Boston, U.S.A. pp 55-58.

Sweeney, C.R. (1989) Tracheal mucus transport rate in healthy horses. *American Journal of Veterinary Research* **50**, 2135 - 2137.

Swinburne, J.E., Bogle, H., Klukowska-Rotzler, J., Drogemuller, M., Leeb, T., Temperton, E., Dolf, G. and Gerber, V. (2009) A whole-genome scan for recurrent airway obstruction in warmblood sport horses indicates two positional candidate regions. *Mamm Genome* **20**, 504-515.

Thornton, D., Carlstedt, I. and Sheehan, J. (1996a) Identification of glycoproteins on nitrocellulose membranes and gels. *Molecular Biotechnology* **5**, 171-176.

Thornton, D.J., Carlstedt, I., Howard, M., Devine, P.L., Price, M.R. and Sheehan, J.K. (1996b) Respiratory mucins: Identification of core proteins and glycoforms. *Biochem. J.* **316**, 967-975.

Thornton, D.J., Davies, J.R., Kirkham, S., Gautrey, A., Khan, N., Richardson, P.S. and Sheehan, J.K. (2001) Identification of a nonmucin glycoprotein (gp-340) from a

purified respiratory mucin preparation: Evidence for an association involving the muc5b mucin. *Glycobiology* **11**, 969-977.

Thornton, D.J., Davies, J.R., Kraayenbrink, M., Richardson, P.S., Sheehan, J.K. and Carlstedt, I. (1990) Mucus glycoproteins from 'normal' human tracheobronchial secretion. *Biochem. J.* **265**, 179-186.

Thornton, D.J., Gray, T., Nettesheim, P., Howard, M., Koo, J.S. and Sheehan, J.K. (2000) Characterization of mucins from cultured normal human tracheobronchial epithelial cells. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **278**, L1118-L1128.

Thornton, D.J., Howard, M., Khan, N. and Sheehan, J.K. (1997) Identification of two glycoforms of the muc5b mucin in human respiratory mucus. *J. Biol. Chem.* **272**, 9561-9566.

Thornton, D.J., Rousseau, K. and McGuckin, M.A. (2008) Structure and function of the polymeric mucins in airways mucus. *Annu. Rev. Physiol.* **70**, 459-486.

Thornton, D.J., Sheehan, J.K., Lindgren, H. and Carlstedt, I. (1991) Mucus glycoproteins from cystic fibrotic sputum. Macromolecular properties and structural 'architecture'. *Biochem. J.* **276**, 667-675.

Tremblay, G.M., Ferland, C., Lapointe, J.M., Vrins, A., Lavoie, J.P. and Cormier, Y. (1993) Effect of stabling on bronchoalveolar cells obtained from normal and copd horses. *Equine Vet J* **25**, 194-197.

Vairo, S., Van den Broeck, W., Favoreel, H., Scagliarini, A. and Nauwynck, H. (2013) Development and use of a polarized equine upper respiratory tract mucosal explant system to study the early phase of pathogenesis of a european strain of equine arteritis virus. *Vet Res* **44**, 22.

Van der Sluis, M., De Koning, B.A.E., De Bruijn, A.C.J.M., Velcich, A., Meijerink, J.P.P., Van Goudoever, J.B., Broller, H.A., Dekker, J., Van Seuningen, I., Renes, I.B. and Einerhand, A.W.C. (2006) Muc2-deficient mice spontaneously develop colitis, indicating that muc2 is critical for colonic protection. *Gastroenterology* **131**, 117-129.

Vandekerckhove, A., Glorieux, S., Broeck, W.V.d., Gryspeerdt, A., van der Meulen, K.M. and Nauwynck, H.J. (2009) In vitro culture of equine respiratory mucosa explants. *The Veterinary Journal* **181**, 280-287.

Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., Kucherlapati, R., Lipkin, M., Yang, K. and Augenlicht, L. (2002) Colorectal cancer in mice genetically deficient in the mucin muc2. *Science* **295**, 1726-1729.

Vinall, L.E., Fowler, J.C., Jones, A.L., Kirkbride, H.J., de Bolos, C., Laine, A., Porchet, N., Gum, J.R., Kim, Y.S., Moss, F.M., Mitchell, D.M. and Swallow, D.M. (2000) Polymorphism of human mucin genes in chest disease: Possible significance of muc2. *Am J Respir Cell Mol Biol* **23**, 678-686.

Vinall, L.E., Hill, A.S., Pigny, P., Pratt, W.S., Toribara, N., Gum, J.R., Kim, Y.S., Porchet, N., Aubert, J.-P. and Swallow, D.M. (1998) Variable number tandem repeat polymorphism of the mucin genes located in the complex on 11p15.5. *Human Genetics* **102**, 357-366.

Voynow, J.A., Fischer, B.M., Malarkey, D.E., Burch, L.H., Wong, T., Longphre, M., Ho, S.B. and Foster, W.M. (2004) Neutrophil elastase induces mucus cell metaplasia in mouse lung. In: *100th International Conference of the American-Thoracic-Society*, Amer Physiological Soc, Orlando, FL. pp L1293-L1302.

Voynow, J.A., Young, L.R., Wang, Y.Q., Horger, T., Rose, M.C. and Fischer, B.M. (1999) Neutrophil elastase increases muc5ac mrna and protein expression in respiratory epithelial cells. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* **276**, L835-L843.

Vrins, A., Doucet, M. and Nunez-Ochoa, L. (1991) A retrospective study of bronchoalveolar lavage cytology in horses with clinical findings of small airway disease. *Zentralbl Veterinarmed A* **38**, 472-479.

Wagner, B. (2009) Ige in horses: Occurrence in health and disease. *Vet Immunol Immunopathol* **132**, 21-30.

Wickstrom, C. and Carlstedt, I. (2001) N-terminal cleavage of the salivary muc5b mucin. *J. Biol. Chem.* **276**, 47116-47121.

Wickstrom, C., Christersson, C., Davies, J.R. and Carlstedt, I. (2000) Macromolecular organization of saliva: Identification of 'insoluble' muc5b assemblies and non-mucin proteins in the gel phase. *Biochem. J.* **351**, 421-428.

Wickstrom, C., Davies, J.R., Eriksen, G.V., Veerman, E.C. and Carlstedt, I. (1998) Muc5b is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: Identification of glycoforms and c-terminal cleavage. *Biochem. J.* **334**, 685-693.

Widdicombe, J.H. and Pecson, I.S. (2002) Distribution and numbers of mucous glands in the horse trachea. *Equine Vet. J.* **34**, 630-633.

Willoughby, R.A., Ecker, G.L., McKee, S.L. and Riddolls, L.J. (1991) Use of scintigraphy for the determination of mucociliary clearance rates in normal, sedated, diseased and exercised horses. *Can J Vet Res* **55**, 315-320.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L. and Donaldson, D.D. (1998) Interleukin-13: Central mediator of allergic asthma. *Science* **282**, 2258-2261.