

**ASSESSMENT OF A METHODOLOGY FOR DETERMINATION OF
H₂O₂ CONCENTRATION AND pH IN EXHALED BREATH
CONDENSATE IN HORSES WITH AND WITHOUT LOWER AIRWAY
INFLAMMATION**

by

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ABSTRACT

Measurement of hydrogen peroxide (H_2O_2) concentration and pH in exhaled breath condensate (EBC) is useful for detection and monitoring of diseases of the lower respiratory tract in humans, including asthma and chronic obstructive pulmonary disease. In contrast, limited information on the use of these parameters for the investigation of lower airway inflammation (LAI) is available for horses. Aims of the current study were to develop a device to collect EBC from non-sedated horses, assess inter-day and intra-day variability of EBC H_2O_2 concentration and pH and determine if there was an association between these parameters and the presence and severity of LAI. The methodology used for collection of EBC was adapted from that used in a previous study (Wyse *et al.* 2005) and was evaluated for its repeatability. Hydrogen peroxide was measured using previously reported analytic methods (Wyse *et al.* 2005). During studies for the determination of a methodology for measurement of pH in EBC, samples of EBC were divided into 3 aliquots. One aliquot was left non-deaerated, one aliquot was deaerated with argon and one aliquot underwent standardisation with CO_2 . The pH of each aliquot was measured using a bench pH meter. The most repeatable measurement of EBC pH was obtained from plain samples (i.e. non-deaerated). Intra-day and inter-day variability of H_2O_2 concentration and pH were assessed by collecting and analysing EBC three times a day for 3 consecutive days. For H_2O_2 the variability in concentrations was large (60-103%), while the pH of EBC had little variability (3-7%). No significant difference in the intra-day or inter-day H_2O_2 concentrations or pH measurements were found in control or LAI horses, except for inter-day H_2O_2 concentration in horses with LAI ($p=0.019$). There was no significant difference in EBC H_2O_2 concentration between control horses (Mean \pm SD) and horses with LAI (mean \pm SD). There was no significant difference in EBC pH between control horses (Mean \pm SD) and horses with LAI (mean \pm SD), however a trend for a reduced pH in horses with LAI was observed ($p=0.079$). The results of this study suggest that EBC H_2O_2 concentration is

not suitable for detecting mild LAI or monitoring horses with LAI due to the high variability. The pH of EBC is more stable overtime and the measurements are more repeatable than H₂O₂ concentration and further studies are required to improve the stability of its measurement and to assess its sensitivity in a population of horses with more severe LAI against healthy controls. Further studies are also required to determine whether determination of H₂O₂ concentration in EBC is useful for detection of marked LAI in horses.

AUTHORS DECLARATION

I, Marco Duz, declare that the work in this thesis is original, was carried out solely by myself or with due acknowledgments. It has not been submitted in any form for another degree of professional qualification.

Marco Duz

Part of this thesis has been accepted for publication elsewhere:

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TABLE OF CONTENTS

<i>Abstract</i>	Pg. <i>ii</i>
<i>Authors Declaration</i>	<i>iv</i>
<i>Acknowledgments</i>	<i>v</i>
<i>Table of contents</i>	<i>vi</i>
<i>List of figures</i>	<i>viii</i>
<i>List of tables</i>	<i>x</i>
<i>List of abbreviations</i>	<i>xii</i>
 CHAPTER 1 – INTRODUCTION	 1
CHAPTER 2 – LITERATURE REVIEW	5
2.1 Role of Phagocytosis on Oxidative Stress	5
2.1.1 <i>Oxidizing Agents in Phagocytosis</i>	5
2.1.2 <i>Oxidative Stress</i>	7
2.1.2.1 <i>Oxidative stress in the airways</i>	9
2.2 Exhaled Breath Analysis	11
2.2.1 <i>Collection Methods: Bias and Limitations</i>	13
2.2.1.1 <i>Human studies of exhaled breath condensate collection</i>	13
2.2.1.2 <i>Equine studies of exhaled breath condensate collection</i>	16
2.2.2 <i>Hydrogen Peroxide Concentration In Exhaled Breath Condensate</i>	17
2.2.2.1 <i>Usefulness of measurement of EBC H₂O₂ in human medicine</i>	18
2.2.2.2 <i>Limitations of measurement of EBC H₂O₂</i>	20
2.2.3 <i>Exhaled Breath Condensate pH</i>	22
2.2.3.1 <i>Usefulness of measurement of EBC pH in human medicine</i>	23
2.2.3.2 <i>Limitations of measurement of EBC pH</i>	25
2.3 Lower Airway Inflammation in Horses	27
2.3.1 <i>Recurrent Airway Obstruction (Heaves)</i>	28
2.3.2 <i>Summer -Pasture-Associated Chronic Obstructive Disease</i>	35
2.3.3 <i>Inflammatory Airway Disease (IAD)</i>	36
2.4 Diagnosis of LAI in Horses	39
2.4.1 <i>Collection of History and Clinical Signs</i>	39
2.4.2 <i>Airway Endoscopy, Cytology and Bacteriology</i>	40
2.4.3 <i>Lung Function Testing</i>	42
2.4.4 <i>Other Imaging Techniques</i>	44
2.4.5 <i>Haematology and Serum Biochemistry</i>	45
2.4.6 <i>Environmental Challenges and Managements</i>	45
2.4.7 <i>Immunological Testing</i>	46
2.4.6 <i>Exhaled Breath Condensate Analysis in Horses</i>	46
Aims of the Study	49
CHAPTER 3 - MATERIALS AND METHODS FOR COLLECTION AND ANALYSIS OF EBC FROM HORSES	50
3.1 Collection of EBC	50
3.1.1 <i>Construction of the Face Mask</i>	50
3.1.2 <i>Condensation Chamber</i>	52
3.1.3 <i>Cleaning the Equipment</i>	53
3.1.4 <i>Animals</i>	54
3.2 Measurement of EBC H ₂ O ₂ concentration and pH	55
3.2.1 <i>Measurement of EBC H₂O₂ Concentration</i>	55
3.2.1.1 <i>Spectrophotometric assay</i>	55
3.2.1.2 <i>Lower detection limit and limit of quantification</i>	56
3.2.2 <i>Measurement of EBC pH</i>	56
3.2.2.1 <i>Introduction</i>	56
3.2.2.2 <i>Materials and methods</i>	57
3.2.2.3 <i>Results</i>	59
3.2.2.4 <i>Discussion</i>	60
3.3 Repeatability of the Methodology for Collection and Analysis of EBC H ₂ O ₂ Concentration and pH	63

3.3.1 Introduction	Pg. 63
3.3.2 Materials and Methods	64
3.3.3 Results	65
3.3.4 Discussion	66
CHAPTER 4 – STUDY OF INTRA-DAY AND INTER-DAY VARIABILITY IN EBC	
H₂O₂ CONCENTRATION AND pH	70
4.1 Introduction	70
4.2 Materials and Methods	71
4.2.1 Animals	71
4.2.2 Collection of EBC Samples and Analysis	73
4.2.3 Study Design	73
4.2.4 Statistical Analysis	73
4.3 Results	74
4.4 Discussion	79
CHAPTER 5 – EBC H₂O₂ CONCENTRATION AND pH IN HEALTHY HORSES AND	
HORSES WITH LAI	85
5.1 Introduction	85
5.2 Materials and Methods	87
5.2.1 Animals	87
5.2.2 Collection of EBC Samples and Analysis	91
5.2.4 Statistical Analysis	91
5.3 Results	91
5.4 Discussion	97
CHAPTER 6 – GENERAL DISCUSSION AND CONCLUSIONS	102
6.1 Collection and Analysis of EBC in Horses	102
6.2 Future Use of EBC H ₂ O ₂ Analysis in the Horse	108
6.3 Future Use of EBC pH Analysis in the Horse	109
6.4 Conclusions	111
LIST OF REFERENCES	114
APPENDIX 1 – List of manufacturers	134
APPENDIX 2 – List of abbreviations	135
APPENDIX 3 – Figures of equipment for EBC collection	137
APPENDIX 4 – Figures of laboratory analysis of EBC	141

LIST OF FIGURES

Figure 3.1: Photograph of equipment used to collect exhaled breath condensate from a mature horse. The airtight face mask (A) is attached to flexible rubber tubing (B) that is connected to the condensation chamber immersed in an ethanol/liquid nitrogen slurry (C). The system includes two one-way non-rebreathing valves incorporated into the face mask (D) and a one-way valve at the outlet of the condensation chamber (E) to prevent contamination of the sample with environmental air.	Pg.51
Figure 3.2: Schematic representation of the system used to collect exhaled breath condensate from horses. Exhaled breath is conveyed to the condensation chamber (magnified version on the right) by means of the one-way valve and rubber tubing. The internal shape of the chamber was designed to direct the flow of expired breath towards the bottom of the PP container	53
Figure 3.3: pH of exhaled breath condensate over time in plain samples, after Argon deaeration and CO ₂ standardisation. The pH was measured at each time point (1,3,5,7 and 10 minutes) during deaeration and when the pH stabilised ("final") after discontinuation of treatment with Argon or CO ₂ . For the plain EBC samples, pH was measured every 2-3 minutes after until a stable reading was obtained.	60
Figure 4.1: Box and whiskers quartile chart of H ₂ O ₂ concentration in EBC of control horses (n=11) collected on 5 occasions over a 54 hour period to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30hr and 54 hr); *=mean	78
Figure 4.2: Box and whiskers quartile chart of the pH of EBC of control horses (n=11) collected on 5 occasions over a 54 hour period to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30 hr and 54 hr). No significant difference was present (p=0.299); *=mean.	78
Figure 4.3: Lack of correlation between EBC H ₂ O ₂ concentration and pH in the horses of the control group (Pearson's correlation coefficient=-0.127; p=0.354)	79
Figure 4.4: Lack of correlation between EBC H ₂ O ₂ concentration and pH in the horses of the LAI group (Pearson's correlation coefficient=-0.277; p=0.181)	79
Figure 5.1: Boxplot of EBC H ₂ O ₂ concentration for each group; no statistically significant difference between groups was present (p=0.895); *= mean	94
Figure 5.2: Boxplot of EBC pH of the pasture control group, stable control group and lower airway inflammation group; no significant difference between the three groups was detected (p=0.188); *= mean	94
Figure 5.3: Distribution of H ₂ O ₂ concentration and pH in EBC of healthy horses; there was no correlation between H ₂ O ₂ concentration and pH (Pearson's correlation coefficient=-0.205; p=0.372)	94
Figure 5.4: Distribution of H ₂ O ₂ concentration and pH and trend line in horses with lower airway inflammation; no correlation was detected (Pearson's correlation coefficient=0.183; p=0.665)	94
Figure 5.5: Relationship between respiratory rate and EBC pH in healthy horses; a significant relationship was detected using a Pearson's correlation coefficient (0.631; p=0.002)	97

APPENDIX 3 - Figure 1: the facemask used in the study was build from a plastic muzzle adapted with fibreglass and resin. Two openings were used to attach one-way valves	Pg.137
APPENDIX 3 - Figure 2: inner view of the mask where two holes were left open and modelled with fibreglass and artificial resin to fit the attachment of the one-way valves	137
APPENDIX 3 - Figure 3: A rubber ring was used to establish an air-tight connection between each one-way valve and the mask	138
APPENDIX 3 - Figure 4: the rubber shroud sealed the mask on the muzzle of the horse creating a closed unidirectional circuit preventing contamination of the exhaled breath sample with ambient air and permitting generation of negative pressures necessary to activate the one-way valves	138
APPENDIX 3 - Figure 5: One-way valves used in the study. Two valves were connected to the mask. Each valve has an internal diameter of 3.5cm and ensure a unidirectional flow of air during respiration by the horse	139
APPENDIX 3 - Figure 6: A) The connection of the face mask to the condensation chamber was obtained through flexible rubber/plastic tubes; B) The device is used in the standing non-sedated horse	139
APPENDIX 3 - Figure 7: The condensation chamber has a one way valve (A) at the end of the system to isolate of from the environmental air. The PP chamber (B) in immersed in the ethanol/liquid nitrogen slurry	140
APPENDIX 4 - Figure 1: A) The Horse Radish Peroxidase (HRP) was available as a powder and was reconstituted as a solution by adding 1.36ml of distilled water for every mg of powder; B) The TMB (3,3',5,5'-tetramethylbenzidine) used was available in 1mg tablets and was prepared in solution mixing 1mg of TMB with 0.5ml of Acetone, 0.5ml of methanol and 9ml of 0.42M citrate buffer solution (pH 3.84)	141
APPENDIX 4 - Figure 2: A) Microplate ready for analysis with standard concentrations (0, 0.1, 0.5, 1, 5 and 10 μ M) on the left and samples of EBC on the right. B)The spectrophotometer read the absorbance of each well (FLUOstar OPTIMA, BMG labtech, UK) at the wavelength of 450nm	141
APPENDIX 4 - Figure 3: A) Electrochemical bench pHmeter and B) electrode (6mm diameter) used to measure the pH of exhaled breath condensate in sample as little as 0.3ml	142

LIST OF TABLES

Table 3.1: Values of pH in plain aliquots of EBC, aliquots of EBC deaerated with Argon and CO ₂ in four horses	Pg. 59
Table 3.2: Mean, standard deviation (SD) and coefficient of variation (CV) values for measurement of pH in plain aliquots of EBC and aliquots of EBC deaerated with argon or CO ₂	59
Table 3.3: Results of the study of the repeatability of the collection and analysis methodology for EBC H ₂ O ₂ and pH. Exhaled breath condensate pH, H ₂ O ₂ concentration, respiratory rate and volume collected for each sample and the coefficients of variation for pH and H ₂ O ₂ repeated measurements are reported. For horse 2 am not enough sample was available and H ₂ O ₂ concentration was measured only	65
Table 4.1: Clinical and bronchoalveolar lavage cytology data from 5 horses with lower airway inflammation	72
Table 4.2: Mean values (±SD) of all the measurements of H ₂ O ₂ concentration and pH from the control and LAI groups	75
Table 4.3: Coefficient of variation values for intra-day and inter-day measurements of both H ₂ O ₂ concentration and pH in exhaled breath condensate from 11 horses without evidence of lower airway inflammation (control group)	75
Table 4.4: Coefficient of variation values for intra-day and inter-day measurements of H ₂ O ₂ concentration and pH in exhaled breath condensate of the 5 horses in the LAI group	75
Table 4.5: Intra-day and inter-day EBC H ₂ O ₂ concentration and pH collected on 5 occasions over a 54 hour period from the horses in the control group (n=5) to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30hr and 54 hr)	75
Table 4.6: Intra-day and inter-day EBC H ₂ O ₂ concentration and pH collected on 5 occasions over a 54 hour period from the horses in the LAI group (n=5) to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30hr and 54 hr). *= statistically significant (p=0.019)	75
Table 5.1: Signalment, stable bedding used and feeding of control horses housed in stables	88
Table 5.2: Signalment and housing of pastured control horses	89
Table 5.3: Signalment, history, clinical signs and BALF cytology of horses with lower airway inflammation (LAI group)	90
Table 5.4: Mean and standard deviation (SD) of respiratory rate and volume of EBC collected from the Stable control group, Pasture control group and lower airway inflammation (LAI) group	92
Table 5.5: Mean and SD of H ₂ O ₂ concentration and pH of EBC collected from the Stable control group, Pasture control group and lower airway inflammation (LAI) group	92
Table 5.6: EBC H ₂ O ₂ concentration and pH, respiratory rate (RR; breaths per minute) and volume of EBC collected from the Stable control group, Pasture	

control group and horses in the group with lower airway inflammation (LAI) (*: horses 9 and 13 were excluded for detection of abnormal tracheal sound during auscultation)

Pg.93

Table 5.7: Pearson's correlation coefficient for variables in healthy horses; *:statistically significant

96

Table 5.8: Pearson's correlation coefficient for variables in horses with lower airway inflammation

96

CHAPTER 1 - INTRODUCTION

A number of conditions are associated with lower airway inflammation (LAI) in horses, including recurrent airway obstruction (RAO), summer-pasture-associated RAO (SPA-RAO) and inflammatory airway disease (IAD) (Dixon *et al.* 1995a; Couetil *et al.* 2007). Recurrent airway obstruction occurs in mature horses that are stabled and is characterised by accumulation of mucus, bronchospasm and neutrophilia in the lower airways and reversible airway obstruction. Signs include cough, mucopurulent nasal discharge, abnormal tracheal and lung sounds, decreased performance, increased respiratory effort or respiratory distress. The pathogenesis and clinical signs of SPA-RAO are similar to RAO, however the disease is more common in horses at pasture and prevalence is greater during summer. In contrast, horses with IAD do not exhibit increased respiratory effort at rest and signs of disease tend to be confined to cough, nasal discharge and poor performance (Couetil *et al.* 2007). Inflammatory airway disease is most common in young racehorses, however horses of any age or breed can be affected (Couetil *et al.* 2007).

The etiopathogenesis of LAI involves the inhalation of aerosolised environmental irritants and/or antigens and development of airway inflammation, however the mechanisms of disease remain to be established. Recurrent airway obstruction likely results from specific hypersensitivity reactions to fungal aeroallergens (McGorum and Dixon 1993; McGorum *et al.* 1993a), however evidence suggests a role for non-antigen specific inflammatory responses (i.e. to endotoxins, β -glucans) (Pirie *et al.* 2001; Pirie *et al.* 2003). The etiopathogenesis of IAD remains poorly defined and a variety of respirable irritants and antigens may be involved. The involvement of environmental factors in the development of RAO and IAD has been reinforced by studies demonstrating that

introduction to a stable environment is a risk factor for development of LAI in horses (Robinson 2001; Malikides 2003; Couetil *et al.* 2007).

Diagnosis of LAI in horses is achieved by diagnostic imaging and/or cytological examination of secretions obtained from the lower airways. Endoscopy is useful to detect and semi-quantify the presence of mucus within the larger airways. Collection and analysis of a tracheal aspirate (TA) is often used for investigation of lower respiratory tract disease. Tracheal aspirate samples are used commonly for cytological and bacteriological examinations. However, the usefulness of TA cytology has been questioned by Derksen *et al.* (1989) who found no correlation between the cytological results of TA and BAL samples in a mixed population of horses and suggested that TA cytology does not provide information on the health of the lower airways. Conversely, Christley *et al.* (2001) in a study performed using Thoroughbred racehorses, documented a strong correlation between signs of respiratory tract disease (cough) and neutrophil percentage in TA samples, suggesting TA cytology may be useful in more homogenous populations. Cytological examination of bronchoalveolar lining fluid (BALF) obtained by BAL is used commonly to confirm the presence of airway inflammation. However, the BAL procedure is invasive, usually requires sedation of the animal, frequently induces transient signs of discomfort during the procedure and can induce neutrophilic airway inflammation (Sweeney *et al.* 1994). Consequently BAL is not suitable for frequent sampling to monitor horses with LAI and alternative methods are required.

Exhaled breath condensate (EBC) has been studied widely in human patients with inflammatory diseases of the lower airways, including asthma and chronic obstructive pulmonary disease (COPD) (Jobsis *et al.* 1997; Ho *et al.* 1999; Hanazawa *et al.* 2000; Niimi *et al.* 2004; Brunetti *et al.* 2006). Collection of EBC is non-invasive, easy to perform and can be

repeated frequently as samples are not influenced by previous sampling. Numerous compounds are present in EBC from humans including hydrogen peroxide (H_2O_2), nitric oxide, adenosine, arachidonic acid metabolites, cytokines, 8-isoprostane and ammonia (Horvath *et al.* 2005). Hydrogen peroxide concentration in EBC is considered a suitable analyte for frequent monitoring of humans with respiratory disease (Loukides *et al.* 1998; Loukides *et al.* 2002; Horvath *et al.* 2005). Hydrogen peroxide is produced primarily by activated inflammatory cells in the airways as a consequence of oxidative stress (Babior 2000). In humans, H_2O_2 is also produced by epithelial cells in the conducting airways and expiratory flow rate may influence its concentration in EBC (Schleiss *et al.* 2000). Potential limitations to the use of H_2O_2 are that it is photo- and thermo-sensitive, extremely reactive towards a wide selection of materials and subject to rapid degradation. A wide inter-day coefficient of variation for EBC H_2O_2 in healthy humans has been reported (van Beurden *et al.* 2002a) and may be due to either chemical reactivity and instability or circadian rhythms of H_2O_2 secretion (Nowak *et al.* 2001). In contrast, other authors described good correlation between EBC H_2O_2 concentration and the presence and severity of asthma and response to treatment (Antczak *et al.* 2000; Loukides *et al.* 2002). Similarly, EBC pH has been demonstrated to be a robust and reliable indicator of inflammatory respiratory diseases in humans (Kostikas *et al.* 2002; Vaughan *et al.* 2003; Borrill *et al.* 2005).

Knowledge of the usefulness of EBC H_2O_2 concentration and pH (including physiological values) in the investigation of LAI in horses is limited. Deaton *et al.* (2004a) described good correlation between the presence and severity of respiratory disease and H_2O_2 concentration in EBC in horses with RAO. However, no significant increase of H_2O_2 concentration in horses with RAO exposed to environmental challenge compared to control horses was found in other studies (Deaton *et al.* 2005a; Wyse *et al.* 2005). Exhaled breath condensate analysis, including determination of

H₂O₂ concentration and pH, could represent an alternative to BALF cytology and allow frequent assessment of horses with LAI. However, further validation of the methodologies for collection analysis of EBC and understanding of the physiologic variations in EBC H₂O₂ and pH in the horse are required.

The aims of the present thesis were to:

1. describe a technique for collection of EBC from horses
2. describe methodologies for determination of H₂O₂ concentration and pH of EBC obtained from horses
3. determine the intra-day and inter-day variability in EBC H₂O₂ concentration and pH in a group of horses
4. apply the use of EBC collection and analysis (H₂O₂ concentration and pH) for detection of LAI in horses.

CHAPTER 2 – LITERATURE REVIEW

2.1 Role of Phagocytosis on Oxidative Stress

2.1.1 Oxidizing Agents in Phagocytosis

Phagocytes, in particular neutrophils and macrophages, are an integral component of innate immune responses. These cell types produce a series of oxidizing agents to digest and destroy phagocytised particles including pathogens and non-infectious material. The oxidizing agents are very efficient in damaging pathogens, however adjacent healthy tissues can be injured also, particularly with protracted inflammatory responses.

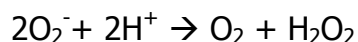
Baldrige (1933) first described a transiently increased consumption of oxygen by neutrophils and macrophages exposed to bacteria. This process was named the “respiratory burst” and the increased uptake of oxygen was initially attributed to an increased phosphorylation in the mitochondria to provide the required energy necessary for the phagocytosis (Baldrige 1933). Thirty years later, the theory of mitochondrial participation was questioned by the discovery that H_2O_2 was produced using oxygen by the stimulated phagocytes, thereby explaining the increased oxygen uptake when these cells were in the presence of an inflammatory stimulus (Iyer *et al.* 1961).

There are four principal oxidizing agents present in the phagosomes of phagocytes: *hydrogen peroxide* (H_2O_2), *nitric oxide* (NO), *superoxide* (O_2^-) and *hypochlorous acid* (HOCl). These oxidising agents are produced by a series of reactions and each mechanism is catalysed by a specific enzyme (Babior 2000).

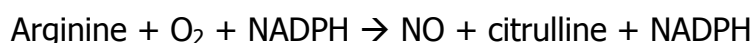
The first of these reactions is catalysed by the membrane-bound enzyme *NADPH oxidase* and produces O_2^- from oxygen and NADPH:



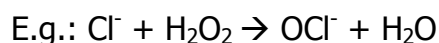
Second, *Superoxide dismutase* catalyses the conversion of O_2^- to H_2O_2 and oxygen:



The reaction between arginine, oxygen and NADPH is catalysed by *nitric oxide synthase* to produce nitric oxide (NO), citrulline and NADPH:

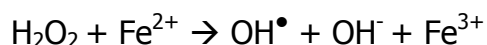


Last, *Myeloperoxidase* uses H_2O_2 , produced from the dismutation of O_2^- , for the oxidation of halide ions (Cl^- , Br^- , I^-) to hypohalous acids:

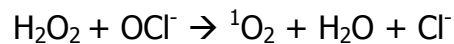


Free radicals are compounds that contain one or more unpaired electrons and can be generated by non-enzymatic reactions with other free radicals. A common feature of free radicals is that they lead to the generation of another free radical by reacting with a molecule with no unpaired electrons. A large variety of free radical species can be generated by reaction with carbon, oxygen, nitrogen or sulphur.

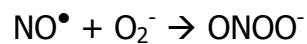
The formation of reactive oxygen and nitrogen species (RONS) is a consequence of aerobic metabolism. A number of RONS generating systems are present in the body, and are involved in the physiological redox modulation of the intracellular environment (Dosek *et al.* 2007). One of the most common free radicals is hydroxyl radical (OH^\bullet) that originates from the reaction between H_2O_2 and metals such as iron or copper in a low valence state. Other secondary products (e.g. Fe^{3+} , OCl^-) are a group of reactive oxidants produced in phagosomes:



Singlet oxygen ($^1\text{O}_2$) is very reactive as the two electrons present in the O_2 molecule are paired. This free radical is believed to be responsible for some of the damage to the phagocytised particles in neutrophils and is usually produced by the reaction between hydrogen peroxide and an oxidized halogen:



Reactive nitrogen species such as peroxynitrite (ONOO^-) are also commonly produced from the reaction of nitric oxide (NO) with O_2^- :



All these oxidants are well contained in the phagosome and contribute to the digestion of the phagocytised particles, however they may be released with the death of the phagocyte, inducing damage to the surrounding tissues.

2.1.2 Oxidative Stress

Oxidative stress is a recognized mechanism involved in physiological processes, such as aging (Donato *et al.* 2007; Lee and Wei 2007) and also many pathological events such as asthma and COPD in humans (Marshall 2001). It is usually present where there is an imbalance between the production of free radicals and antioxidants, such that there is a lack in protection against the oxidative damage induced by free radicals. Oxidative stress can damage many cellular components and contribute to the development of a wide variety of diseases including inflammatory processes (Ercan *et al.* 2006) and cancer (Toyokuni 2006).

The cellular redox environment is preserved by enzymes that maintain it in a reduced state. A constant input of metabolic energy is required for the maintenance of enzyme function and the reduced redox state. Disturbances in the normal redox state may induce toxic effects to the cell

through the production of peroxides and free radicals that can damage the lipids of the cytoplasmic membrane, DNA and cellular proteins (Bentinger *et al.* 2007).

Oxidative stress can be detected by quantifying free radicals or the products of the effect of these oxidants on cell components in body tissues or fluids. Markers of lipid peroxidation include 8-isoprostane, an indicator of oxidation of arachidonate via a non-enzymatic pathway (Psathakis *et al.* 2004) and ethane that originates from the reaction between RONS and lipids in cellular membranes. Lipid peroxidation has been studied in great detail and the mechanism is well characterized (Paredi *et al.* 2000).

Protein oxidation, induced by free radicals, involves specific aminoacids as not all aminoacids are sensitive to oxidation. Oxidation usually begins with a reaction between a hydroxyperoxide protein-bound transition metal (iron or copper) resulting in hydroxyl radicals at the metal binding site (Stohs and Bagchi 1995; Gaetke and Chow 2003). Hydroxyl radicals oxidize individual amino acids and the subsequent reactions that occur are similar to lipid peroxidation (Lapenna *et al.* 2005). The propagation phase of oxidation can continue within the protein, to another protein or to a lipid. Oxidized proteins are often hydrolyzed back to amino-acids by specific proteases (Lapenna *et al.* 2005).

Oxidation of DNA, elicited by RONS, takes place in a similar manner to that of proteins and requires the presence of a bound transition metal. In the nucleus there are two possible pathways available for repairing the damage; removal and replacement of the appropriate nucleotide or removal of oligomers of oxidized bases that are replaced by DNA polymerase and sealed by DNA ligase (Bentinger *et al.* 2007).

Endogenous defence mechanisms against oxidation are present in the body and usually involve molecules broadly referred to as antioxidants. These molecules terminate chain reactions started by oxidizing agents by removing radical intermediates and by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or phenols. Antioxidants may be enzymatic (including superoxide dismutase, glutathione peroxidase and catalase) or non-enzymatic (including ascorbic acid, vitamins E, vitamin B complex, carotenes and glutathione). A recent study in humans has also described the first-line role of Coenzyme Q; this enzyme has been shown to protect from the oxidation of free radicals both at the level of the cytoplasmic membrane and of DNA in the nucleus (Navas *et al.* 2007).

2.1.2.1 Oxidative stress in the airways

Physiological and pathological conditions that are induced by or induce oxidative stress in the airways have been described in human studies. High altitude environments are characterised by lower oxygen tension in the atmosphere and induce oxidative stress in the airways; exercise exacerbates this process even in healthy and fit individuals (Araneda *et al.* 2005). The intranasal administration of oxygen can induce oxidative stress by increasing the amount of oxygen available to be transformed to reactive oxygen forms (Carpagnano *et al.* 2006). In addition, patients with chronic respiratory diseases (i.e. chronic obstructive pulmonary disease (COPD), asthma, bronchiectasis or cystic fibrosis) and smokers are more likely to develop oxidative stress than healthy non-smokers (Kostikas *et al.* 2003; Horvath *et al.* 2005).

Many studies have analysed the role of oxidative stress in airway disease by quantifying the levels of exhaled biomarkers of oxidative stress in patients with different pathological conditions compared to matched

controls. These studies have led to ongoing developments in exhaled breath analysis, with wide application in patients with respiratory disease. Further benefit is derived from the non-invasive nature of EBC collection and high patient compliance.

Studies of the role of oxidative stress in the lower airways of horses have been performed (Deaton *et al.* 2005a; Deaton *et al.* 2005b; Deaton 2006) and described an association between the concentration of ascorbic acid in BALF, EBC H₂O₂ concentration and presence and severity of clinical signs of respiratory disease in horses with RAO. Decreased concentration of ascorbic acid in BALF and an increased EBC H₂O₂ concentration in RAO-affected horses was suggestive that oxidative stress is likely to be involved in the pathogenesis of RAO (Deaton *et al.* 2004a).

Oxidative damage to cellular lipids is known as lipid peroxidation and results in the production of pentane and ethane gas. Detection of these hydrocarbons in the exhaled breath has been proposed to be useful parameters to monitor oxidative stress in the airways of horses (Wyse *et al.* 2004; Wyse *et al.* 2005). Further, exercise alone is known to be sufficient to induce oxidative stress in healthy horses (Wyse *et al.* 2005).

On the basis of a limited number of studies (Deaton *et al.* 2004b; Deaton *et al.* 2005b; Wyse *et al.* 2005; Deaton *et al.* 2006) oxidative stress is likely to be related to the presence and severity of RAO in horses. A greater understanding of oxidative stress in the lower airways of horses could lead to useful and non invasive methods to detect and monitor LAI diseases in horses, including RAO, SPA-RAO and IAD.

2.2 Exhaled Breath Analysis

Analysis of exhaled breath (EB) and exhaled breath condensate (EBC) in human respiratory medicine has undergone rapid and promising scientific development during the last decade. Early studies, published in the 1990s, demonstrated the usefulness of these procedures in problematic patients, such as children, that were not suitable candidates for traditional diagnostic procedures such as broncho-alveolar lavage (BAL) (Scheideler *et al.* 1993). The non-invasive nature of sample collection prompted the development of a wide range of tests for detection of compounds in EBC and/or EB that may serve as markers of LAI. Oxidative stress in the airways can lead to cellular damage and death and subsequent release of many different molecular products of oxidation into the airways, which are then removed from the airways by expiration (Battaglia *et al.* 2005; Gerritsen *et al.* 2005). Methodologies of EB and EBC analysis are based on the identification and quantification of molecules that are thought to be specific indicators of oxidative stress in the lower airways associated with pulmonary pathology. Exhaled breath is composed of a gaseous phase and a water vapour-saturated phase (Horvath *et al.* 2005). Many substances have been identified in the gaseous phase of breath including nitric oxides, adenosine, arachidonic acid and derivatives, cytokines (eg. interleukins), stable prostaglandin-like products (eg. 8-isoprostanes, aldehydes, ammonia (NH_3) and ammonium (NH_4^+) and various anions (ATS/ERS 2005; Horvath *et al.* 2005). The possible roles of several of these molecules as markers of inflammation and disease have been investigated in humans. Nitric oxide, carbon monoxide (CO), carbon dioxide (CO_2) and ethane have been studied most extensively and their usefulness in the investigation of respiratory disease in humans has been demonstrated and reported (Kharitonov *et al.* 1997; de Gouw *et al.* 1998; Horvath *et al.* 1998; Kroesbergen *et al.* 1999; Montuschi *et al.* 2001; American Thoracic and European Respiratory 2005).

The water vapour-saturated phase of EB can be collected as EBC. Greater than 99% of EBC is water and the remaining is a combination of hydrophobic and hydrosoluble non-volatile molecules (Effros *et al.* 2002). The mechanisms by which the smallest respiratory particles are transferred to the exhaled gases are not understood and further studies are required to better understand whether disease conditions may interfere with this process (Horvath *et al.* 2005). In humans, H₂O₂ concentration and the pH of EBC have been evaluated in a large number of studies involving patients with various respiratory diseases and healthy controls. The concentration of H₂O₂ in EBC is well correlated to the presence of several respiratory diseases such as asthma, COPD, bronchiectasis and acute respiratory distress syndrome (ARDS) (Kietzmann *et al.* 1993; Jobsis *et al.* 1997; Jobsis *et al.* 1998; Loukides *et al.* 2002; Kostikas *et al.* 2003). Patients with asthma have higher concentrations of H₂O₂ in EBC compared to healthy individuals (Loukides *et al.* 2002). Asthmatic patients also have been shown to have higher concentrations of H₂O₂ in EBC during asthma exacerbation compared to periods of remission of the disease (Loukides *et al.* 2002). Studies of the pH of EBC have also been reported and provide a greater understanding of the involvement of airway acidification in the pathogenesis of lower airway inflammation (Loukides *et al.* 1998; Loukides *et al.* 2002; Vaughan *et al.* 2003; Borrill *et al.* 2005; Brunetti *et al.* 2006).

Although many publications have described EBC collection, few studies have assessed methodological factors that may affect EBC collection and analysis. Methods of collection and analysis vary between studies and comparison of results between studies that do not use the same protocol is difficult. Differences in methods of collection, assays used for the determination of different substances and inter-lab variations due to the use of different equipment can all potentially bias the results and need to be described in detail in each study (Horvath *et al.* 2005).

2.2.1 Collection Methods: Bias and Limitations

2.2.1.1 Human studies of exhaled breath condensate collection

The American Respiratory Society/European Thoracic Society Task Force has developed guidelines for EBC collection and analysis of different biomarkers and have made recommendations for future developments (Horvath *et al.* 2005). The drafting of specific and strict guidelines for EBC collection was avoided, as the lack of validation studies hampered evidence-based recommendations (Horvath *et al.* 2005). Since the substances investigated in EBC have different biochemical structures and properties, different collection techniques may affect the half-life of different substances in the sample. Therefore, the choice of a particular methodology of collection should be guided by the biochemical properties of the substance of interest. Volatile substances, such as ammonia, tend to leave the sample soon after collection and need to be analysed immediately, while more stable substances, such as cytokines and arachidonic acid metabolites, can be frozen and stored for long periods of time without alteration in concentration (Horvath *et al.* 2005).

One common factor between the different methodologies described in the literature is exhaled breath is directed by a one way flow into a collection system containing a condensation chamber kept at low temperature (usually -80°C) that allows the condensation of the exhaled vapours in the breath and retention of the EBC in the condensation chamber. In human studies, several commercially available devices have been developed to improve the standardization of EBC collection. The RTube™ (Respiratory Research Inc. VA) is a commercially available and disposable device that allows collection of EBC at home according to the patients' schedule, or during exacerbations of respiratory disease when a sample may be more clinically significant. A mouthpiece is connected through a one-way valve directing exhaled breath into a polypropylene (PP) collection tube

surrounded by an aluminium sleeve cooled to -20°C before collection. The circuit also contains a trap to avoid contamination of the sample with saliva. The patient breathes through the inlet one-way valve of the collection system for a pre-determined amount of time and then stores the sample obtained at <0°C (i.e. in a domestic freezer with temperature of approximately -20°C), until it is analysed. Studies that used this device to collect EBC have benefited from the feasibility of home collection making large population studies possible as well as frequent repeated collections from the same patients (Vaughan *et al.* 2003; Paget-Brown *et al.* 2006). A second available device is the ECoScreen (Jaeger, Wurzburg, Germany). With this device, exhaled breath enters and leaves the chamber through a one-way, non-rebreathing valve which prevents mixing of inspiratory and expiratory gases and filters saliva. This method uses an electrical refrigeration device that moves exhaled air through a lamellar condenser with a sample collection vial inserted into a cooling cuff. The interior temperature is constantly maintained at less than -15°C.

Since the RTubeTM and the ECoScreen are the most commonly used devices in published studies, a direct comparison between the two methods has been performed to determine whether results obtained are comparable (Soyer *et al.* 2006). The temperature in the condensation chamber of the ECoScreen was found to be more stable and lower than that of the RTubeTM. However, the main advantages of the RTubeTM, including transportability and low cost, make it useful for collection of EBC and analysis of the compounds in EBC that are minimally influenced by a rise in temperature. These advantages make this device suitable for use in studies of large populations. Another difference between these two devices is that the valve of the RTubeTM is made of silicone rubber whereas that of the lamellar condenser of the ECoScreen is made of aluminium coated with teflon. It has been suggested that some mediators, such as cytokines and other proteins, may be recovered more efficiently

from Teflon surfaces compared to silicon rubber, however little evidence is available to support this statement (Soyer *et al.* 2006; Prieto *et al.* 2007).

Another device available commercially is the TURBO-DECCS (ItalChill, Parma, Italy) which has been used to study the effect of temperature of collection on the concentration of hydrogen peroxide in EBC of healthy individuals (Goldoni *et al.* 2005). The working temperature is adjustable from -10°C to room temperature or higher. This system allows a very accurate control of the collection temperature and therefore is ideal for studies aimed at investigating the effect of temperature on concentration and stability of different substances collected within the EBC (Goldoni *et al.* 2005).

The use of custom-made devices with a condensation chamber usually constituted of inert materials (either glass or Teflon), has been described by several authors (Kietzmann *et al.* 1993; Horvath *et al.* 1998; Mutlu *et al.* 2001; van Beurden *et al.* 2002b; De Benedetto *et al.* 2005). Although the results of these studies were similar to results of other studies using commercially available devices, it is possible that the unique nature of those systems may have influenced the results obtained or at least limited the repeatability of sample collection by other authors.

Human EBC is collected through mouth expiration and most devices have been designed to prevent sample contamination with saliva. These systems are usually referred as saliva traps and are essential as production of saliva by some human patients can be marked. An accepted recommendation is to look for salivary contamination if a saliva trap is not utilized by measuring the salivary amylase (Effros *et al.* 2002). The use of nose clips to minimise contaminations of EBC samples with nasal secretions has been suggested by some authors, however there is insufficient data to currently support the use of these devices (Horvath *et*

al. 2005). Neither saliva traps or nose clips are applicable for collection of EBC from horses due to obligate nasal breathing in this species.

Duration of EBC collection, contamination of the materials in contact with EBC and possible interactions between materials used and substances in the EBC sample are mandatory considerations when planning a study. The recommendation of the American Respiratory Society and of the European Thoracic Society is to record the duration of each collection and that this parameter should be set for each study protocol (Horvath *et al.* 2005). Furthermore, all materials that are in contact with EBC should be tested to exclude contamination of the sample that may confound results. The pH of EBC can be affected by contamination of the samples with saliva and the systems of collection and storage should be assessed to detect any salivary contamination (Horvath *et al.* 2005).

2.2.1.2 Equine studies of Exhaled breath condensate collection

Two devices have been described in the scientific literature for EBC collection from horses (Deaton *et al.* 2004b; Wyse *et al.* 2005). Between the two studies there were differences in equipment design. The device used by Deaton *et al.* (2004b) included a U-shaped stainless steel condensing tube that contained an inner volume of 5 litres. The condensing tube was immersed in ice and cold water to ensure a surface temperature for the condensation of EB of approximately 0°C. The tubing connecting the condensing tube to the horse was placed inside a larger and warmer pipe to keep the temperature in the inner pipe at a sufficiently high temperature to avoid condensation in the connecting pipe and maximize sample retrieval. Collection of EBC from horses was obtained by a modified AeroMask (Equine AeroMask™, Trudell Medical International, Veterinary Division, London, Ontario, Canada) that included a rubber shroud to provide an airtight seal on the face of the horse and a

one-way valve at the attachment of the connecting pipe to the condensation pipe. Wyse *et al.* (2005) used a Pyrex glass condensation chamber immersed in liquid nitrogen/ethanol slurry to create a much colder environment ($<-80^{\circ}\text{C}$) for the collection of EBC than that used by Deaton *et al.* (2004b). Collection of EBC was obtained through a face mask sealed with a rubber shroud on the muzzle of the horse and unidirectional airflow was obtained with a non-rebreathing valve. The connecting pipes were made of rubber but were not warmed, to increase the portability of the device. Differences in equipment and temperature used for EBC collection and between the studies of Deaton *et al.* (2004b) and Wyse *et al.* (2005) limit the comparison of the results of these two studies.

2.2.2 Hydrogen Peroxide Concentration in Exhaled Breath Condensate

Of the substances present in EBC, hydrogen peroxide has been studied most extensively. In the respiratory system, H_2O_2 is thought to be produced by inflammatory cells (predominantly neutrophils, macrophages and eosinophils) and epithelial cells in the conducting airways (Schleiss *et al.* 2000). A relationship between H_2O_2 concentration in EBC and the presence and severity of respiratory diseases in humans including asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, cystic fibrosis and pulmonary effects of smoking has been established (Jobsis *et al.* 1997; Horvath *et al.* 1998; Jobsis *et al.* 1998; Loukides *et al.* 1998; Kostikas *et al.* 2003).

Differences in number and activation of inflammatory cells in the lower airways may explain the reported relationship between H_2O_2 and disease severity. Several studies have been performed in an attempt to identify which specific cell types produce H_2O_2 and the site of production in the

airways of humans with various diseases of the lower respiratory tract (Kietzmann *et al.* 1993; Montuschi *et al.* 2001; Nowak *et al.* 2001; Effros *et al.* 2002). Flow dependency of H₂O₂ concentration in EBC has been demonstrated in healthy and mildly asthmatic humans suggesting that production of H₂O₂ also occurs in the conducting airways and not only from the distal airways (Jobsis *et al.* 1997). The consistent increase in H₂O₂ concentration at lower respiratory flow rates supports the suggestion that there is a major bronchial contribution in addition to alveolar production (Schleiss *et al.* 2000). Schleiss *et al.* (2000) concluded that if alveolar H₂O₂ concentration is high, H₂O₂ could be absorbed within the bronchi during expiration and consequently the amount of exhaled H₂O₂ will decrease with decreasing flow rates. Therefore monitoring expiratory flows during collection of EBC may contribute to the interpretation of EBC H₂O₂ concentration (Schleiss *et al.* 2000; Horvath *et al.* 2005).

2.2.2.1 Usefulness of measurement of EBC H₂O₂ in human medicine

Hydrogen peroxide is commonly present in EBC obtained from healthy humans and concentrations are variable, depending on age and living habits. Elderly people have higher concentrations than young adults and healthy children, but lower concentrations than smokers and ex-smokers. Patients with pulmonary disease including COPD (van Beurden *et al.* 2002a), bronchiectasis (Loukides *et al.* 1998) and acute respiratory distress syndrome (ARDS) (Kietzmann *et al.* 1993) have statistically significantly higher H₂O₂ concentrations in EBC compared to controls. Children and adults with asthma have a greater concentration of EBC H₂O₂ than healthy individuals (Jobsis *et al.* 1997; Horvath *et al.* 1998). Further, a correlation with the severity of disease in asthmatic humans has been described; EBC H₂O₂ concentrations increase during exacerbations of the disease, while clinical improvement usually leads to a decrease in EBC

H₂O₂ concentration (Loukides *et al.* 2002). Conversely, studies of patients with cystic fibrosis failed to demonstrate increased concentrations of H₂O₂ in EBC. This is possibly due to the degradation of the majority of H₂O₂ through reaction with viscous airways secretions or other reactive species or increased antioxidant activity caused by trapping of positively charged antioxidants in negatively charged airways secretions (Ho *et al.* 1999). The use of other markers in EB, for example CO is more appropriate in the investigation of cystic fibrosis (Kharitonov and Barnes 2002).

The potential usefulness of EBC in monitoring the effect of treatment in patients with respiratory disease has been investigated. In mildly asthmatic patients a relationship between H₂O₂ concentrations and response to treatment with corticosteroids have been documented, whereby a treatment with inhalatory HFA-beclomethasone for 4 weeks significantly decreased EBC H₂O₂ concentrations for a period of at least two weeks after the discontinuation of the medication concurrent with remission of symptoms (Antczak *et al.* 2000). In patients with clinically stable COPD, treatment with N-acetylcysteine produced similar results, significantly reducing EBC H₂O₂ concentration after only 15 days of treatment (Kharitonov and Barnes 2004; De Benedetto *et al.* 2005).

While considerable advances in the analysis of EBC and EB obtained from humans has occurred over the last decade, these methodologies and analytes are not considered suitable for sole monitoring of patients with respiratory disease and further data are warranted to better understand the potential of these tests in human respiratory medicine (Horvath *et al.* 2005). The applicability of these methodologies in routine clinical settings is still limited. Studies on the physiology of EBC formation and its dilution markers and improvements in the reproducibility of these tests are still warranted before they can substitute more traditional methods in the management of individual patients (Horvath *et al.* 2005).

2.2.2.2 Limitations of measurement of EBC H₂O₂

Although several authors have outlined the potential of determination of EBC H₂O₂ concentration for use in human respiratory medicine (Jobsis *et al.* 1997; Horvath *et al.* 1998; Loukides *et al.* 1998; Kostikas *et al.* 2003) and to a lesser degree in horses (Deaton *et al.* 2004b; Wyse *et al.* 2005), measurement of H₂O₂ is not without certain considerations. Being a free radical, H₂O₂ is a very reactive molecule that can undergo rapid degradation during interaction with different materials (Horvath *et al.* 2005). Because of this instability, samples of EBC must be analysed immediately or frozen and stored at temperatures $\leq -70^{\circ}\text{C}$ until analysis of H₂O₂ concentration (Horvath *et al.* 2005).

In humans, the concentration of H₂O₂ is up to 100 fold greater in saliva than in EBC and it is therefore mandatory to exclude any salivary contamination of obtained EBC to ensure the sample is representative of the lower respiratory tract (Horvath *et al.* 2005). Many of the devices used for collection of EBC from humans in previously published studies contained saliva traps to avoid sample contamination (Horvath *et al.* 2005). Alternatively, EBC samples from humans may be collected during nasal respiration to exclude contamination with saliva, however H₂O₂ concentrations are not comparable with those obtained by oral collection (Latzin *et al.* 2003).

The laboratory method used for determination of H₂O₂ concentration in EBC can influence the obtained results. Many methods for measurement of H₂O₂ concentration have been reported and the physiological concentrations of EBC H₂O₂ are often very close to, or even less than, the lower detection limit of many of these assays (about 0.1 $\mu\text{m/L}$) (Gallati and Pracht 1985; van Beurden *et al.* 2002b; Svensson *et al.* 2004). Colorimetric or fluorimetric assays are most commonly used. Automated

fluorimetric assays are most accurate and precise and have detection limit of 0.005 $\mu\text{m/L}$ (van Beurden *et al.* 2002b). An automated flow injection analysis with fluorescence detection has similar detection limit (40nm/L) but these methods require the use of equipment with limited availability (Svensson *et al.* 2004).

For any diagnostic test, a large coefficient of variation for repeated measurements makes comparison of data within and between studies difficult. Assessment of variation of H_2O_2 concentration in EBC of human patients has been performed by determining both the intra-day and inter-day coefficient of variation (CV) of EBC H_2O_2 concentration using repeated sample collection from the same patients (van Beurden *et al.* 2002a). In the study of van Beurden (2002a) analysis of three samples of EBC collected on the same day from healthy non-smoking individuals revealed an intra-day coefficient of variation (CV) of H_2O_2 concentration that was approximately 20%. In addition, the inter-day CV from four samples collected over a period of 21 days was 43% (van Beurden *et al.* 2002a). The same protocol was used also in a group of patients with clinically stable COPD and the calculated CV were similar to the control group (approximately 24% and 45% for intra-day and inter-day repeated measurements respectively) (van Beurden *et al.* 2002a). A significant and repeatable increased production of H_2O_2 at 12am and 12pm was also described and was interpreted by the authors as circadian variation (van Beurden *et al.* 2002a).

Nowak *et al.* (2001) assessed diurnal variations in H_2O_2 concentration in EBC using a protocol similar to that described by van Beurden *et al.* (2002a). Intra-day variation in H_2O_2 concentration was assessed by collecting four samples of EBC in a single day from 58 healthy volunteers and inter-day variation was determined by collection of three further samples every seven days during two consecutive weeks from the same

individuals. In that study, EBC H_2O_2 concentrations were greater at 12 am and 12 pm and lower at 8 am and 8 pm. This variation was considered to be dependent on diurnal variation in leukocyte receptors and circulating concentrations of adhesion molecules, changes in antioxidant enzyme activity or differing concentrations of low molecular weight antioxidants in epithelial lining fluids (Nowak *et al.* 2001).

The duration of EBC collection has been studied as a possible cause for variability in H_2O_2 concentration, however no significant difference in EBC H_2O_2 concentration was found when samples obtained over a collection period of 4 or 10 minutes from human patients were compared (Svensson *et al.* 2004).

To date none of the published studies of EBC collection from horses (Deaton *et al.* 2004b; Wyse *et al.* 2005) determined intra-day and inter-day variations in H_2O_2 concentration, or the effect of duration of collection on H_2O_2 concentration.

2.2.3 Exhaled Breath Condensate pH

Exhaled breath condensate pH is considered to reflect the acid-base balance status of the airways and can be easily measured in samples obtained from human patients. The normal homeostasis between acid and basic buffer systems is the result of balanced production and release of acids and bases in the airways. Changes in airway pH have been associated with many pathological events and a low pH of EBC is thought to be a consequence of inflammation in the airways (Kostikas *et al.* 2002). Airway acidification has been implicated in the pathophysiology of asthma as it accelerates necrosis of human eosinophils and the conversion of endogenous nitrites to NO (Hunt *et al.* 2000). Exhaled breath condensate pH values have been found to be identical to those obtained from lower

airway secretions, thus reflecting the acidification in the lower respiratory tract (Hunt *et al.* 2000). However, it is not entirely understood if endogenous acidification is an unequivocal indicator of the presence of inflammation in the airways (Hunt *et al.* 2000). A reduction in airway pH has been shown to induce bronchoconstriction, impairment of ciliary motility, increased mucus viscosity and damage to airway epithelium. Measurement of EBC pH is usually obtained using bench pHmeters that have high accuracy and are sensitive to changes of ± 0.01 pH units (Horvath *et al.* 2005). Blood gas analysers with similar accuracy have also been used recently (Kullmann *et al.* 2007).

2.2.3.1 Usefulness of measurement of EBC pH in human medicine

As with many other physiologic variables, pH in EBC has been studied widely in order to understand how it is influenced by respiratory diseases. The pH of samples from patients with asthma, COPD, bronchiectasis and healthy controls have been measured, to establish the magnitude of change occurring in these diseases (Hunt *et al.* 2000; Tate *et al.* 2002; Borrill *et al.* 2005; Horvath *et al.* 2005; Brunetti *et al.* 2006). Correlation between EBC pH and a variety of inflammatory airway conditions in humans has been shown, including eosinophilic and neutrophilic inflammation in induced sputum (Kostikas *et al.* 2002). While the pH of EBC from patients with mild asthma was not different from that of healthy controls, more severe forms of the disease induced a significant decrease in pH (Kostikas *et al.* 2002). The same authors reported that in general, patients with bronchiectasis complicated by chronic infections with *Pseudomonas aeruginosa* had lower pH values than asthmatic patients (Kostikas *et al.* 2002). However, Hunt *et al.* (2000) reported that the lowest EBC pH values were in samples from patients with acute episodes of asthma. In another study, children with allergic rhinitis and/or atopic dermatitis had an EBC pH lower than healthy controls and it was

suggested that the more acidic environment in the lower airways of these patients could predispose to development of asthma and monitoring of EBC pH could be useful for early detection of this condition (Brunetti *et al.* 2006).

The relationship between lower airway inflammation and EBC pH is indicated further by the finding that inhalatory corticosteroid therapy in human patients with LAI results in an increase in the pH of EBC (Hunt *et al.* 2000). Hence measurement of the pH of EBC may be useful to monitor patients receiving treatment with corticosteroids in a non-invasive manner, as a decrease in activity of inflammatory cells and reduced production of molecules that can induce acidification in the lower airways would be expected (Kostikas *et al.* 2002; Horvath *et al.* 2005; Brunetti *et al.* 2006).

Acidification of EBC in patients with cystic fibrosis has been recorded, likely due to reduced excretion of buffers (eg. bicarbonates) in the lower airways (Tate *et al.* 2002). Airway acidification may be important in the pathogenesis of this disease, as it may reduce ciliary function, increase mucus viscosity and impair some humoral immune mechanisms (Tate *et al.* 2002).

A large study of healthy non-smoking human patients was performed to assess the intra-weekly and intra-daily variations in EBC pH and whether different methodologies of collection could bias the data (Vaughan *et al.* 2003). The CV for intra-week and intra-day repeated measurements in that study were 4.5% and 3.5%, respectively. Collecting at different temperatures, collection times and ventilatory patterns did not significantly alter the pH in the samples obtained. Furthermore, storage for prolonged periods of time (>12 months) had a minimal effect on the CV of pH measurement (3%). The pH of EBC has been found to be very

similar before and after induction of general anaesthesia in human patients scheduled for elective surgical procedures (Wells *et al.* 2005). In that study collection of exhaled breath samples was achieved through a cuffed tracheal tube, therefore bypassing the upper respiratory tract. In EBC obtained from healthy human patients the pH is slightly alkaline and within a narrow range (Wells *et al.* 2005). The findings of Wells *et al.* (2005) and other studies (Vaughan *et al.* 2003; Borrill *et al.* 2005; Bloemen *et al.* 2006) indicate that EBC pH is a simple, robust and reproducible indicator of acidification in the lower airways.

2.2.3.2 Limitations of measurement of EBC pH

The pH of EBC tested immediately after sampling from healthy human subjects tends to be unstable and obtained measurement may be unreliable (Kullmann *et al.* 2007). To reduce the instability of EBC pH, attempts have been made to stabilize the samples of EBC. Carbon dioxide is one of the main contributors to pH instability in EBC and is the major volatile component of EBC (Kullmann *et al.* 2007). In an aqueous environment, CO₂ reduces the pH by forming H⁺ and HCO₃⁻ through the chemical reaction: $\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$. Procedures to remove CO₂ from EBC are referred to as deaeration or gas-standardization and are achieved by bubbling a CO₂-free gas through the sample for a set time period (Kullmann *et al.* 2007). The rationale for deaeration of EBC is to control the variation resulting from the interaction between EBC and the atmosphere. Argon is reported to be used most frequently, as it is a noble gas and is unlikely to interact with any of the endogenous substances in the sample (Horvath *et al.* 2005). Alternatively, nitrogen or oxygen have been used for gas-standardisation (Horvath *et al.* 2005). Some authors have preferred to measure the pH of EBC immediately after collection without any deaeration (Tate *et al.* 2002; Bloemen *et al.* 2006). Deaeration of EBC samples obtained from healthy humans and humans

with asthma using argon resulted in good repeatability of pH measurements, with CV of approximately 3.3% for both groups (Hunt *et al.* 2000). Furthermore, deaerated samples had values of pH identical to EBC samples suctioned directly from the lower airways (Vaughan *et al.* 2003). In human patients with COPD undergoing mechanical ventilation, a close relationship between pH measurements before and after Argon deaeration has been reported (Gessner *et al.* 2003). However, other authors have shown that the effect of gas standardisation is unpredictable for samples with an initial pH lower than 6, possibly due to inefficient removal of CO₂ or to the presence of strong acids in the EBC. Complete removal of CO₂ is not possible as a residual amount is always present after deaeration, potentially confusing pH assessment (Borrill *et al.* 2006; Horvath *et al.* 2006).

Recently, a study of the effect of CO₂ partial pressure on EBC pH was reported (Kullmann *et al.* 2007). This procedure consisted of preparing the EBC samples with CO₂, achieved by bubbling CO₂ in the sample for 1 second and then pH and EBC CO₂ partial pressure (P_{EBC,CO₂}) were measured with a blood gas analyser. The data obtained were used to calculate the value of the pH at 5.33 kPa P_{EBC,CO₂} (physiological P_{EBC,CO₂}) in the lower airways by data regression analysis. Because this methodology accounted for the variability of the pH measurement due to the variable amount of CO₂ present in the sample, results were approximately six times more repeatable than deaeration with argon (Kullmann *et al.* 2007). Further investigations are required to validate the methodology; however the study of Kullmann *et al.* (2007) proved that CO₂ has a major role in influencing EBC pH.

Other influences on the pH measured from EBC samples include contamination with endogenous substances of oral origin and patient activity. While contamination of EBC samples with ammonia from the oral

cavity has been suggested to influence EBC pH there is little evidence that it interferes with the total EBC pH (Effros *et al.* 2003; Horvath *et al.* 2005). Recently, moderate exercise was shown to induce an increase of pH in healthy patients compared to the pre-exercise values (Riediker and Danuser 2007). Therefore exercise may affect the sensitivity of EBC pH by masking the acidification induced by an ongoing inflammatory process (Riediker and Danuser 2007) and has ramifications for timing of EBC sample collection from patients in relation to exercise.

2.3 Lower Airway Inflammation in Horses

Disorders of the lower respiratory tract are common in horses and can affect animals of all ages and breeds. Presenting complaints may include exercise intolerance, cough, nasal discharge, fever, respiratory distress increased respiratory rate or effort, signs of depression or inappetence. Diseases that can affect the airways and/or the parenchyma of the lungs include bacterial, viral, parasitic or fungal pneumonia, lung abscess, pleuropneumonia, acute respiratory distress syndrome (ARDS), pulmonary oedema, exercise induced pulmonary haemorrhage (EIPH), interstitial lung disease, recurrent airway obstruction (RAO), summer pasture-associated recurrent airway obstruction (SPA-RAO), inflammatory airway disease (IAD), smoke inhalation and neoplasia. These diseases are associated with inflammation of the lower airways and/or lung parenchyma and different mechanisms are involved dependent on aetiology and disease process. Recurrent airway obstruction, SPA-RAO and IAD involve specific and/or non-specific immune responses to antigens that enter the lower airways through inhalation. These diseases are collectively referred to as lower airway inflammation (LAI) in this thesis. Clinical signs of lower airway disease are not specific and a definitive diagnosis is frequently difficult to achieve on clinical examination alone. The use of ancillary diagnostic techniques including endoscopy,

tracheal aspirate cytology, bronchoalveolar lavage (BAL) cytology, thoracic radiography, ultrasonography, arterial blood gas analysis, pulmonary function testing may provide information on the ongoing process, contribute to obtaining a definitive diagnosis and provide information on disease severity. Further, these tests may provide evidence of the pathophysiology of different disease processes that affect the lower airways (Ainsworth and Hackett 2004).

Lower airway inflammatory disorders of horses have important implications for the horse industry. Diseases such as RAO, SPA-RAO or pneumonia can be invaliding and cause obvious poor performance and loss of athletic potential and premature retirement from athletic activities (Robinson 2001). While other pulmonary diseases may not result in any clinical abnormalities or rest (eg. IAD, EIPH), impaired lung function during exercise may result in exercise intolerance or poor performance (Hinchcliff *et al.* 2005; Couetil *et al.* 2007).

2.3.1 Recurrent Airway Obstruction (Heaves)

Recurrent airway obstruction is a common inflammatory disease of the lower airways of mature horses (Dixon *et al.* 1995a; Robinson 2001). Although there are many studies describing the clinical signs attributable to RAO, the relationship between causal factors, structural changes, airway hyper-reactivity and airway inflammation remain incompletely understood. Recurrent airway obstruction is characterised by reversible airway narrowing due to bronchospasm that is exacerbated by mucosal hypertrophy and intraluminal secretions. The most frequent clinical sign of RAO is cough that can occur during/after exercise, or after exposure to triggering factors contained in mouldy hay or dusty environments. Many horses with RAO also have a mucous to mucopurulent bilateral nasal discharge. The respiratory pattern of affected horses can also change as

bronchospasm increases the resistance to the airflow in the distal airways, principally during expiration. With protracted disease, hypertrophy of abdominal muscles subsequent to the increased respiratory effort may occur resulting in the characteristic heave line, from which the name "heaves" is derived. For many years RAO has been compared to human asthma because of common features such as airway hyper-responsiveness and reversible airway narrowing. Cytologically one difference between RAO and asthma is neutrophils are the dominant cell type of the epithelial lining fluids of the lungs in RAO while eosinophils may predominate in human asthma. Previously RAO was referred to as chronic obstructive pulmonary disease (COPD), a term derived from human respiratory medicine. However, many differences are recognized between RAO and human COPD, the latter being a progressive disease with little or no reversibility of airway constriction, often related to smoking habits. Currently the accepted terminology is recurrent airway obstruction (RAO) known also as "heaves" or "broken wind" (Robinson *et al.* 2003). The microscopic anatomy of the airways in horses with RAO has been described in detail using light and electron microscopy (Kaup *et al.* 1990a; Kaup *et al.* 1990b; Pirie *et al.* 1990). Principal abnormalities of the alveolar region include focal necrosis of type I epithelial cells, fibrosis, type II epithelial cell transformation and emphysema with hyperinflation (Kaup *et al.* 1990a). Necrotic foci are usually located in the peribronchiolar region. In the bronchiolar epithelium, the non-ciliated bronchiolar epithelial cells, known also as Clara cells, are the cell type that show the most significant ultrastructural degeneration (Kaup *et al.* 1990a). Metaplasia of the mucus secreting cells (goblet cells) in the lower airway epithelium of horses with RAO has also been described (McPherson and Thomson 1983; Nyman *et al.* 1991). Bronchiolitis, airway smooth muscle hypertrophy, excess mucus and inflammatory cells in the small airways are commonly present in horses with RAO and likely contribute to airway narrowing ante-mortem (Kaup *et al.* 1990a). Macroscopically, the lungs of horses with RAO may

appear hyperinflated and indentations may be present due to the pressure on the thoracic cage in advanced cases (Kaup *et al.* 1990a). In the larger conducting airways, epithelial cell hyperplasia, loss of ciliated cells, mucus accumulation due to decreased clearance, goblet cell hyperplasia and infiltration of neutrophils, eosinophils and mast cells may be present (Kaup *et al.* 1990b).

Much attention has been directed toward the etiopathogenesis of RAO and postulated causes include specific immune-mediated responses to fungal antigens and non-specific inflammatory responses to inhaled pro-inflammatory agents including moulds, endotoxins, particulate and noxious gases which are present in the breathing zone of stabled horses (McPherson *et al.* 1979; McGorum and Dixon 1993; McGorum *et al.* 1993a, d; McGorum *et al.* 1998; Schmallenbach *et al.* 1998; Pirie *et al.* 2001; Pirie *et al.* 2002, 2003; Ward and Couetil 2005). Clinical signs of RAO can be induced by exposure of susceptible animals to dusty and mouldy hay and it has been postulated that RAO is an allergic (type I and/or type III and type IV hypersensitivity) reaction to *Faenia rectivirgula* (*Microspolyspora faeni*), *Aspergillus fumigatus* and/or *Thermoactinomyces vulgaris*, which are abundant in poor quality hay (McGorum *et al.* 1993d). McGorum *et al.* (1993d) reproduced signs of RAO in susceptible horses after exposure to these fungal antigens, supporting the theory that these antigens play a role in RAO pathogenesis. A classic allergic response following exposure to these agents can be biphasic as demonstrated in humans with allergic airway disease (Durham *et al.* 1984). Similarly to humans, in horse with RAO “early-phase response” starts within minutes from exposure to specific antigens with the activation of mast cells with membrane bound allergen-specific immunoglobulin (IgE) (Halliwell *et al.* 1993). Activated mast cells release pro-inflammatory mediators including histamine that stimulate mucus secretion, vasodilatation, microvascular leakage and airway smooth muscle contraction. Immunoglobulin E is more

abundant in bronchoalveolar secretions of horses with RAO compared to controls, however no differences are present in serum concentrations (Halliwell *et al.* 1993), suggesting that any allergic response is confined predominantly to the lower airways. In humans with asthma the early phase response of the disease induces narrowing of the airway lumen and obstruction of airflow within 1 hour from the initial exposure to the stimuli. However, the early-phase response is clinically silent in horses with RAO (Robinson 2001). The 'late-phase response' occurs later after exposure to aeroallergens because of slower chemotactic recruitment of neutrophils coordinated by T cells through the release of Th2-type cytokines and chemokines. The absence of the early phase response in horses with RAO suggest that a classical type I hypersensitivity is not the central pivot in its pathogenesis. Some authors have postulated that a type III hypersensitivity (Arthus) reaction may be involved in the pathogenesis of RAO, namely a response to an allergen to which the immune-system had been previously sensitised (Halliwell *et al.* 1979), however the lack of precipitating serum antibodies toward the allergens has lead to a dismissal of this hypothesis (Lawson *et al.* 1979).

Respirable endotoxins have been implicated in the pathogenesis of RAO and a dose-dependent neutrophilia after inhalation of endotoxin in healthy horses and horses with RAO has been reported (Pirie *et al.* 2001). After inhalation of endotoxin, both healthy horses and those with RAO had a significantly increased lower airway neutrophilia, however worsening of clinical signs was not observed. The airway neutrophilia of horses with RAO was significantly greater in magnitude than that in healthy horses, (Pirie *et al.* 2001). The lack of clinical signs after challenge with inhaled endotoxin suggests that endotoxins are not the only causative agent and involvement of other agents in the pathogenesis of the disease is likely. Moulds and hay dusts have a higher content of β -D-glucan, and are more likely to induce exacerbation of RAO (Pirie *et al.* 2002). It has been

suggested that endotoxin, β -D-glucans and organic dust particles such as fungal antigens have a synergistic pro-inflammatory involvement in the pathogenesis of RAO (Pirie *et al.* 2003). In addition, noxious gases such as ammonia, hydrogen sulphide and methane have been suggested to induce airway inflammation; despite very low ambient concentrations of these gases, it is possible that horses with RAO are more susceptible (Clarke 1987; Clarke *et al.* 1987).

Bronchospasm is a key feature in RAO, since improvement of clinical signs and pulmonary function can be induced after administration of bronchodilators (Jackson *et al.* 2000). Bronchospasm is mediated by airway smooth muscle contraction likely activated by stimulation of parasympathetic tone and activation of muscarinic receptors. In addition, inflammatory mediators such as serotonin, endothelin-1, histamine and LTD₄ can increase the contractility of smooth muscle cells directly and may contribute to bronchoconstriction in horses with RAO. Further, decreased activity of the inhibitory, non-adrenergic, non-cholinergic neural pathways is thought to contribute to the lack in bronchorelaxation (Robinson 2001).

Airway neutrophilia is the main cytological feature of RAO and large numbers of neutrophils can accumulate in the lower airways within 6 hours after exposure to mouldy hay (Pirie *et al.* 2001). However, the details of the mechanisms responsible for neutrophil activation and recruitment remain poorly understood (Pirie *et al.* 2001). Interleukin-8 (IL-8) is one of the most potent neutrophil-specific chemotactic and activating protein, and an increase in IL-8 is present in horses with RAO after environmental challenge, suggesting the importance of this chemoattractant in RAO pathophysiology (Franchini *et al.* 2000). Neutrophils can also produce and secrete pro-inflammatory cytokines such as myeloperoxidase, TNF- α , IL-1 β , IL-6, MIP-2 and IL-8 which have

chemotactic properties that can further exacerbate the inflammatory process (Joubert *et al.* 2001; Art *et al.* 2006). In healthy horses and horses with RAO in remission that are stabled at pasture, mRNA expression of IL-8 is down-regulated compared to the same horses with RAO during exacerbation of the disease (Joubert *et al.* 2001). In healthy stabled horses, bronchoalveolar neutrophilia may occur in the absence of clinical signs, suggesting that in these animals neutrophils may be in a reduced state of activation (Art *et al.* 2006). In the study reported by Art *et al.* (2006) healthy horses had similar neutrophil ratios but significantly lower myeloperoxidase concentration in BALF compared to horses with RAO in remission.

Lymphocytes, specifically T cells, also contribute to the secretion of cytokines. The T-helper type 2 lymphocytes (Th2) are believed to play a central role in allergic airway inflammation, producing interleukin 4 (IL-4), IL-5, IL-9 and IL-13. Interleukin 4 induces the proliferation and differentiation of both B and T cells, IL-5 is involved in the activation of eosinophils, IL-9 stimulates the production of immunoglobulins and mast cells and IL-13 stimulates the growth of B cells and their production of immunoglobulins, production of other interleukins and inhibits T-helper type 1 lymphocytes (Th1) cells. Conversely Th1 are involved in cell-mediated immunity, releasing interferon (IFN)- γ and IL-2, cytokines that stimulate the growth and differentiation of T cells and release of other cytokines (Lavoie *et al.* 2000; Beadle *et al.* 2002; Cordeau *et al.* 2004; Kleiber *et al.* 2005). It is believed that a balance between Th1 and Th2 responses is important for the modulation of airway inflammation, and the expression of mRNA of Th1 and Th2 cytokines in healthy horses and horses with RAO has been investigated (Lavoie *et al.* 2000; Beadle *et al.* 2002). In human asthma, expression of a Th2-type cytokine response is predominant and it has been postulated that similar process may occur in RAO (Lavoie *et al.* 2000). In horses with RAO increased expression of IL-4

mRNA in leukocytes is associated with worsening of clinical signs (Cordeau *et al.* 2004). Interleukin 4 promotes the development of a Th2 cell dominated immunoresponse and the induction of production of IgE by B-cells (Lavoie *et al.* 2000; Beadle *et al.* 2002; Cordeau *et al.* 2004). However, several studies have provided divergent results and it is still not clear whether RAO results predominantly from a Th-1, Th-2 or mixed Th-1/Th-2 disorder (Ainsworth *et al.* 2003).

Macrophages are also likely to be involved in the pathogenesis of RAO. In RAO affected horses, these cells are more active, with a higher density in the distal airways and therefore may be able to produce more pro-inflammatory mediators to attract neutrophils and stimulate epithelial cells, smooth muscles and goblet cells (Derksen *et al.* 1988; Ainsworth *et al.* 2003; Laan *et al.* 2005; Laan *et al.* 2006).

Another characteristic feature of RAO is abnormal mucus production. Goblet cell hyperplasia and metaplasia may result in an increase in viscosity of mucin, occlusion of the lumen of the smaller airways, decreased cough and mucociliary clearability indices and exacerbation of lower airway obstruction (Dixon *et al.* 1995c; Gerber *et al.* 2000). Altered visco-elasticity of mucus in horses with RAO likely contributes to stasis and accumulation of mucus in the lower airways (Gerber *et al.* 2000). Large amounts of mucus can accumulate in the peribronchiolar alveoli and mineralise. These findings are related to the severity of the clinical signs and are more frequent and extensive in more advanced processes and contribute to the irreversible and progressive decline of the respiratory function in affected individuals (Robinson 2001; Lavoie 2007).

The chronic nature of the inflammatory process in RAO may induce structural changes in the airways linked to smooth muscle hypertrophy and bronchiectasis. This increased smooth muscle mass induces not only

narrowing of the lower airways but also a stronger contraction of those muscles, further contributing to bronchospasm (Lavoie 2007).

2.3.2 Summer Pasture-Associated Recurrent Airway Obstruction

Summer pasture-associated recurrent airway obstruction (SPA-RAO) is a respiratory disease characterised by lower airway inflammation, recurrent airway obstruction and respiratory distress. The disease is most common in the southern regions of the United States, however it has been reported also in the UK (Dixon *et al.* 1995b; Mair 1996). The etiopathogenesis of SPA-RAO is not understood completely; however, hypersensitivity to aeroallergens is considered likely and the disease may share several immunological similarities with RAO. Indeed recurrent airway obstruction and SPA-RAO are not mutually exclusive and horses may be affected by both conditions (Dixon *et al.* 1995a; Mair 1996). Summer pasture-associated recurrent airway obstruction occurs in horses kept at pasture and aerosolised spores and pollens have been incriminated as aetiological factors that precipitate hypersensitivity reactions in the lower airways (Ward and Couetil 2005). Summer pasture-associated recurrent airway obstruction has marked seasonality; prevalence of disease is most frequent during the summer months (Mair 1996). Removing affected animals from pasture during summer months and housing in a dust free environment in stables can reduce the reoccurrence and severity of the disease (Beadle 1983). Relationships between the prevalence of SPA-RAO and rainfall, minimum temperature and total pollen and mould counts in the environment have been documented, suggesting that these environmental factors may contribute to the occurrence of the disease (Ward and Couetil 2005). Epidemiological studies have shown an association of occurrence of SPA-RAO and total pollens counts of *Quercus*, *Fraxinus*, *Morus* and *Acer* spp. during the previous 3 months and fungal spores of *Alternaria* spp and *Cladosporium* spp. during the previous month

in the area where the affected horses lived (Ward and Couetil 2005). However, no increased concentration of pollen specific antibodies in the tracheal secretions of affected horses with SPA-RAO were found in one study and the authors postulated that the disease derives from the ingestion of a pasture derived pneumotoxin (Seahorn *et al.* 1997). Nitric oxide may possibly play a role in amplifying the inflammatory process since the expression of the nitric oxide synthase (iNOS) was greater in bronchial epithelial cells in horses with SPA-RAO compared to healthy subjects (Costa *et al.* 2001).

The pathological features in horses with SPA-RAO are accumulation of mucus, accumulation of degenerated and sloughed epithelial and inflammatory cells (mostly neutrophils) in airway secretions and smooth muscle hypertrophy. Unlike RAO, fibrosis and emphysema are not prominent features of SPA-RAO (Costa *et al.* 2000).

2.3.3 Inflammatory Airway Disease (IAD)

Inflammatory airway disease (IAD) is a poorly understood condition and controversy exists over the definition of this disease. Inflammatory airway disease is typified as a syndrome characterised by cough, accumulation of secretions in the trachea, nasal discharge, poor exercise performance and delayed recovery from exercise (Couetil *et al.* 2007). Cytological examination of BALF from horses with IAD demonstrates airway inflammation most typically neutrophilic, however increased percentages of eosinophils and/or mast cells may be present (Moore *et al.* 1995). Inflammatory airway disease is not characterised by increased respiratory effort, systemic illness or haematological or serum biochemical abnormalities. One study found numbers of CD8-positive lymphocytes were increased in BALF from horses with IAD, in contrast with horses with RAO which had CD4-positive lymphocytes (Moore *et al.* 1995). The

authors concluded that these findings support the theory that IAD does not have an allergic aetiology but is possibly linked to a response to environmental factors or to an infectious agents.

Recently, a consensus statement of the American College of Veterinary Internal Medicine proposed the use of the following minimum criteria to define IAD (Couetil *et al.* 2007):

- *Poor performance, exercise intolerance, or coughing, with or without excess tracheal mucus.*
- *Non septic inflammation detected by cytologic examination of bronchoalveolar lavage fluid (BALF) or pulmonary dysfunction based on evidence of lower airway obstruction, airway hyper-responsiveness, or impaired blood gas exchange at rest or during exercise.*

These authors also proposed some exclusion criteria:

- *Evidence of systemic signs of infection (fever, hematologic abnormalities compatible with infection)*
- *Increased respiratory efforts at rest (i.e., heaves)*

The etiopathogenesis of IAD is complex and not completely understood. Several authors have suggested that an infectious component may be involved (Christley *et al.* 2001; Wood *et al.* 2005; Christley and Rush 2007; Couetil *et al.* 2007). Bacterial species isolated from horses with IAD include *Streptococcus zooepidemicus*, *S. pneumoniae*, *S. suis*, *S. sanguis*, *Pasteurella* spp., *Bordetella bronchiseptica*, *Mycoplasma faelis* and *M. equirhinis*. However, the role of bacteria in IAD remains undefined and it is possible that a primary inflammatory process may predispose to secondary colonization by oropharyngeal flora (Christley *et al.* 2001). Recently, Wood *et al.* (2005) found a statistical relationship between IAD and *S. zooepidemicus* and *S. pneumoniae* tracheal infections. In addition the prevalence and incidence of the disease decreased in older horses, suggesting that acquisition of immunity to bacteria may play a protective role (Wood *et al.* 2005). In horses infected by *Dictyocaulus arnfieldi*

airway eosinophilia may also be present and parasitic pneumonia is considered a differential diagnosis when BALF eosinophilia is present (Couetil *et al.* 2007). Little evidence exists to substantiate a role of viral infection in the pathogenesis of IAD. While one study found administration of the recombinant IFN- α (a cytokine with anti-viral properties) reduced respiratory tract inflammation in racehorses with IAD (Moore *et al.* 2004), other studies failed to detect seroconversion to equine herpes virus (EHV-1 and EHV-4), equine rhinovirus (ERhV-1, ERgV-2), equine influenza virus or adenovirus (Christley *et al.* 2001) in horses with IAD.

Non infectious causes have also been suspected to be involved in IAD etiopathogenesis. Horses of all ages may develop IAD, however it is more common in younger horses, especially racehorses in training and it has been suggested that exercise may be involved in the pathogenesis of the disease. It is possible that during strenuous exercise a greater deposition of particulate matter in the lower airways may be enhanced by frequent deep breathing (Christley and Rush 2007). In addition exercise may contribute to exposure of the lower airways to unconditioned air. In addition, disease is more likely to be detected in racehorses due to detection of poor performance. Inhaled endotoxins may play a role in the etiopathogenesis of IAD. Malikides (2003) found that exposure to respirable endotoxin can induce pulmonary inflammation in healthy horses and the concentration of endotoxin in the breathing zone of young racehorses is associated with the etiopathogenesis of IAD. Noxious gases frequently present in a stable may be involved in causing IAD. Sulfur dioxide, nitrogen dioxide, ozone, carbon monoxide, ammonia, hydrogen sulphide and methane are the most commonly recognized substances in ambient air, however the role of these gases in the development of IAD remains unsubstantiated (Christley and Rush 2007).

2.4 Diagnosis of LAI in Horses

2.4.1 Collection of History and Clinical Signs

Thorough collection of a complete history is important for the correct diagnosis of LAI. Housing and feeding management, recurrence of clinical signs, seasonality and response to previous treatment should be ascertained (Hotchkiss *et al.* 2006). Horses with RAO or SPA-RAO have indistinguishable clinical signs, however differentiation may be achievable by evaluation of the historical information. Prolonged respiratory recovery time after exercise and cough during exercise are common in horses with RAO, SPA-RAO or IAD (Hotchkiss *et al.* 2006). Clinical signs of disease may worsen by stabling in dusty environments or on pasture in summer months depending on the specific disease present (Dixon *et al.* 1995b). Horses with IAD may present with history of coughing and/or exercise intolerance, however signs of respiratory insufficiency at rest are absent (Robinson 2003). Exclusion of other causes of poor performance, such as orthopaedic problems or dynamic upper airway disease, is mandatory during the investigation of horses with IAD (Couetil *et al.* 2001). In horses with RAO or SPA-RAO, nasal discharge and intermittent cough may be noticed by owners, with/without an increase in respiratory effort. Recording the duration of clinical signs of respiratory disease may be useful to identify the chronicity of the disease (Hotchkiss *et al.* 2006). In more chronic cases of RAO, prolonged increased expiratory effort induces hypertrophy of the abdominal muscles ("heave line") (Dixon *et al.* 1995b). Fever is not a typical clinical sign of RAO, and weight loss may be present in more severe cases as increased respiratory effort can result in decreased appetite and increased energy consumption by respiratory muscles (Hotchkiss *et al.* 2006). Some authors found tracheal auscultation after a rebreathing bag test to be more successful for the detection of

abnormal sounds than thoracic auscultation, due to less attenuation of breath sounds at this site (Dixon *et al.* 1995b).

Recurrent airway obstruction and SPA-RAO typically affect horses older than seven years of age, while IAD is more frequent in younger horses. However, older horses may also be affected by IAD (Robinson 2001). Many breeds may be affected by LAI diseases and there is no sex predilection for any LAI disease. Some authors have suggested that genetic factors may predispose to RAO but more data are needed to elucidate the heritability of this disease (Ramseyer *et al.* 2007).

2.4.2 Airway Endoscopy, Cytology and Bacteriology

Endoscopic examination of the lower respiratory tract and cytology of broncho-alveolar and tracheal lining fluids are objective and useful techniques to achieve a diagnosis of LAI in horses (Mair 1996). Endoscopy allows the visualization and semi-quantification of respiratory secretions present in the trachea. Dynamic collapse of the intrathoracic trachea may be visualized during coughing. A positive correlation between the severity of the disease and the volume of mucus/mucopus in the trachea is present in horses with RAO (Dixon *et al.* 1995c; Robinson *et al.* 2003). Cytological examination of tracheal aspirate samples allows determination of differential counts of inflammatory cells. In addition, tracheal aspirate samples can be submitted for quantitative and qualitative bacteriology and virology.

Broncho-alveolar lavage (BAL) in general is a safe procedure and is suitable for assessment of diffuse pulmonary disorders where a single sample obtained from a limited region of the lungs is likely to be representative of the entire lungs (McGorum *et al.* 1993e). Collection of broncho-alveolar lining fluid (BALF) for differential counts of inflammatory

cells in the distal airways is considered necessary for the diagnosis of RAO, SPA-RAO and IAD (Dixon *et al.* 1995a; Couetil *et al.* 2007). Recurrent airway obstruction and SPA-RAO are characterised by an increased neutrophil count in BALF (>25%; normal <5) (Dixon *et al.* 1995c). Inflammatory airway disease is characterised by mild neutrophilia, lymphocytosis and monocytosis, and occasionally eosinophilia, in BALF cytology (Moore *et al.* 1995; Couetil *et al.* 2001; Couetil *et al.* 2007). There is insignificant intra-subject variability in BAL cytology in healthy horses kept in a dusty environment (Gerber *et al.* 2004). In addition, healthy horses stabled in a dusty environment may have a neutrophil count in the BALF of >5% and therefore it is necessary to combine the interpretation of BAL cytology with history and other clinical findings (Robinson 2001). The presence of increased mucus, exfoliated epithelial cells and fungi is highly suggestive of impeded mucociliary clearance or respiration of highly dusty air (Kaup *et al.* 1990b). Recurrent airway obstruction is characterised by a high neutrophilia while in IAD neutrophilia is less prominent (Robinson 2001). In addition, IAD may be associated with eosinophilia and increased count of mast cells (Couetil *et al.* 2007). The value of environmental challenge to discriminate RAO from IAD in affected horses has been suggested, since in IAD affected horses there is no change in the respiratory pattern after the challenge (Couetil *et al.* 2007).

Tracheal aspirate (TA) cytology is considered less reliable to quantify lower airway inflammation as results are not correlated with BALF neutrophil counts or lung histology in horses with RAO (Dixon *et al.* 1995c). Great variability is present in the neutrophil count in TA obtained from healthy individuals (Derksen *et al.* 1989). However, in Thoroughbred racehorses the neutrophil percentage in TA from horses without evidence of respiratory disease was <20%, while coughing horses had neutrophil percentage >20% (Christley *et al.* 2001); therefore TA may still be a

useful diagnostic tool in certain groups of horses. Furthermore, the presence of bacteria in TA in absence of clinical signs consistent with bacterial infection is suggestive of colonization secondary to a defective mucociliary clearance (Dixon *et al.* 1995c).

2.4.3 Lung Function Testing

Measurement of lung function may supply data useful to understand the severity of airway obstruction, allowing an objective monitoring of the patient's progress. The diagnostic value of lung function testing using conventional methods is often limited due to the low sensitivity of the methodology: horses with RAO in clinical remission are not significantly different from normal horses (Robinson *et al.* 2000). However, newer methodologies, such as forced expiration oscillometry or volumetric capnography, have increased the sensitivity compared to conventional techniques (Couetil *et al.* 2000). Because of the requirement of specific equipment and expertise these techniques are used mainly in research settings (Robinson *et al.* 2000).

Tests of lung function are aimed at providing a quantitative assessment of changes in the elasticity of the lung and of the resistance of the non-elastic portion of the respiratory tract (Robinson 2003). Dynamic lung compliance (C_{dyn}) and pulmonary resistance (R_L) are common pulmonary function measurements however they are insensitive to small changes in pulmonary function (Marlin and Deaton 2007). Maximal changes in transpulmonary or pleural pressure (commonly referred as $\Delta P_{pl_{max}}$) is measured directly by placing a catheter in the pleural cavity (Littlejohn and Bowles 1980), or indirectly by means of an oesophageal catheter connected to a pressure transducer that offers an accurate estimate of pleural pressure (Derksen and Robinson 1980). Forced oscillatory mechanics appear to be very sensitive to detect changes in lung function

and are non-invasive as flow and pressures are measured at the nares (Young and Tesarowski 1994). Forced expiration has also been evaluated in horses but is invasive and technically demanding therefore impractical in routine clinical settings (Couetil *et al.* 2001). Tidal breathing flow-volume loops indices are obtained non-invasively from conscious horses wearing a facemask and a pneumotachograph (Art and Lekeux 1988) and are useful indicator of disease severity in RAO (Herholz *et al.* 2003a). Respiratory inductance plethysmography provides a measurement of lung volume by means of recording bands placed around thorax and abdomen (Marlin *et al.* 2002).

Arterial and venous blood gas tensions (P_{aO_2} and P_{aCO_2} ; arterial partial pressures for oxygen and carbon dioxide, respectively) measurement are commonly used in clinical practice and are the best indications of the overall pulmonary gas exchange (Marlin and Deaton 2007). Hypoxemia is a frequent finding in RAO, however hypercapnea is more variable due to lower solubility of CO_2 compared to oxygen. With successful treatment of RAO, P_{aO_2} returns to normal and the blood oxygen content tends to be normal. Therefore, arterial blood analysis is useful for monitoring the progress of treatment of RAO. However, some horses may be hypoxic with no clinical signs (called chronic idiopathic hypoxemia), possibly secondary to alveolar hypoventilation, as increased P_{aCO_2} is commonly present also (Dixon *et al.* 1995a). Respiratory acidosis is rare because of the limited development of hyperapnea (Robinson 2003; Christley and Rush 2007; Couetil *et al.* 2007).

In horses with clinical signs of RAO, $\Delta P_{pl_{max}}$, R_L , peak inspiratory and expiratory airflows and work of respiration are increased while ratio of inspiratory time to total breath time, P_{aO_2} , C_{dyn} are usually decreased (Petsche *et al.* 1994; Dixon *et al.* 1995c). A wide range of normal values for these parameters has been reported in several studies making

interpretation of results from individual test difficult (Petsche *et al.* 1994; Dixon *et al.* 1995c; Herholz *et al.* 2003b). It is also possible that location, temporal, daily and seasonal variations may contribute to differences between results of studies (Robinson 2001).

The prevalence of functional abnormalities in the general population of horses with IAD is unknown. However, lung function testing may help to differentiate IAD from clinical forms of RAO, potentially associated with a more severe lung dysfunction (Christley and Rush 2007).

2.4.4 Diagnostic Imaging Techniques

Radiography is useful in detecting gross abnormalities in the anatomy of the respiratory system. However it is an insensitive methodology for the detection of RAO; a broncho-interstitial pattern may be present in more chronic and severe cases. Some authors described a flattening or concavity of the diaphragm subsequent to alveolar over-inflation, and the presence of single or multiple distended bronchi is indicative of bronchiectasis (Lavoie *et al.* 2004).

Thoracic ultrasonography has not been found to be useful for the diagnosis of LAI, since only the pleural surface can be appreciated and pathology predominantly is associated with airways (Reef *et al.* 1991). Some irregularity may be seen on the surface of the visceral pleura however the importance of this finding is difficult to interpret, as this change is a non-specific finding.

Scintigraphic examination has been used to determine the alveolar clearance in horses in research settings but has found limited application in clinical protocols (Votion *et al.* 1999a). This technique was developed to monitor the ratio of inhalation and perfusion in the lower airways of

horses and a mismatch of inhalation and perfusion was present in horses with RAO. This methodology was found to be more sensitive than lung function testing in detecting early changes in lower airways induced by RAO (Votion *et al.* 1999b).

2.4.5 Haematology and Serum Biochemistry

Haemograms, leukograms and fibrinogen are usually within normal limits in horses with RAO, SPA-RAO or IAD. A mild and inconsistent increase in packed cell volume is a possible finding as the result of an elevated mean erythrocyte corpuscular volume.

2.4.6 Environmental Challenges and Managements

The clinical response to treatment or the response to an environmental challenge has been shown to be a reliable diagnostic tool to identify horses with RAO (McGorum *et al.* 1993d). A positive response is consistent with the onset of clinical signs subsequent to exposure to dusty and mouldy hay or dusts (McPherson *et al.* 1979; McGorum *et al.* 1993b, c; Gerber *et al.* 2000; Gerber *et al.* 2004).

The importance of challenge responses implies a relationship between environmental management and control of disease. It is recognised that reducing the exposure to respirable factors triggering the hypersensitivity-response is the main goal in treating and preventing LAI in horses (McGorum *et al.* 1993d). Reducing exposure to feed-associated dusts, moulds and endotoxins is achieved by soaking hay for adequate periods of time (30-45 minutes) before feeding and gradually introducing a pellet or haylage diet (Ramseyer *et al.* 2007). The type of bedding chosen also influences the quantity of antigens present in the stable environment. Bedding made of wood shavings, cardboard, paper or rubber mats is the

best option since they produce minimal amounts of dusts and moulds, while straw is known to dramatically increase the presence of respirable particles in the air (Woods *et al.* 1993). Using low dust bedding materials have been shown to decrease the respirable dust burden by 97% and to decrease aeroallergen challenge (Woods *et al.* 1993). Ideally, ventilation should be improved, to enhance the removal of airborne particles. If possible, daily access to pasture would be expected to improve airway health of affected horses. Ideally, introducing grazing as the sole source of nutrition by keeping animals on pasture 24h a day is the preferred management for horses with RAO, as exposure to the antigens and endotoxins involved in the etiopathogenesis of the disease is likely to be minimal (Robinson 2001). Stabling horses with SPA-RAO is mandatory, since the factors triggering the clinical signs are present in the pasture, however the stabling management should ensure a low dust environment (Robinson 2001; Christley and Rush 2007; Couetil *et al.* 2007).

2.4.7 Immunological Testing

Intradermal allergen testing is useful in human medicine and other domestic animals (Li 2002). Due to high false positive responses documented in numerous studies, these tests have minimal application in horses with LAI (McGorum *et al.* 1993a; Jose-Cunilleras *et al.* 2001; Lorch *et al.* 2001; Wong *et al.* 2005).

2.4.6 Exhaled Breath Condensate Analysis in Horses

Exhaled breath condensate analysis is a recent addition to respiratory medicine in humans and has been the subject of considerable investigation and application (Horvath *et al.* 2005). The non-invasive nature of sample collection is an advantage of this methodology over conventional methods of diagnostic investigation (eg. collection of TA,

BAL). In veterinary medicine, EBC analysis has been assessed in rodents, rabbits, elephants, swine, calves, dogs, cats and horses (Reinhold *et al.* 2000; Wyse *et al.* 2004; Wyse *et al.* 2005; Zollinger *et al.* 2006).

Exhaled breath condensate has undergone only limited investigation in horses and H_2O_2 has been the component of EBC studied most frequently. Deaton *et al.* (2004) found a relationship between H_2O_2 in the EBC, the antioxidant status of the airways (ascorbic acid concentration) and TA and BALF cytological findings in horses with RAO and healthy controls. Hydrogen peroxide was used as an indicator of the oxidative status of the airways and an inverse correlation between concentrations of ascorbic acid and H_2O_2 was found (Deaton *et al.* 2004a). In addition, a significant relationship was found between the concentration of H_2O_2 in EBC and neutrophil count in BALF, suggestive of a relationship between airway inflammation and EBC H_2O_2 content (Deaton *et al.* 2004b). A further study by this research group did not find a correlation between EBC H_2O_2 concentration and the degree of airway inflammation in a study of environmental challenge in horses with RAO (Deaton *et al.* 2005a). A possible explanation of the lack of association is the antigen exposure may not have been severe enough to cause oxidative stress and an increase in H_2O_2 production (Deaton *et al.* 2005a). Environmental challenge may have been sufficient to cause the neutrophilic inflammation but insufficient to cause oxidative stress and increased H_2O_2 production. In that study some variation in EBC H_2O_2 concentration was present for repeated measurements over a period of 24 hours (Deaton *et al.* 2005a). Considerable variation in EBC H_2O_2 concentration has been described in studies of human patients (van Beurden *et al.* 2002a) and it is possible that a similar degree of variation may occur in horses. Determining the magnitude of any variation in EBC H_2O_2 concentration is necessary to objectively define the usefulness of H_2O_2 as a marker of LAI.

A study performed by Wyse *et al.* (2005) involved stabling a group of horses in two different management systems (high vs low dust). A tendency of EBC H_2O_2 concentration to increase in the high dust management was found, however the difference between groups was not statistically significant (Wyse *et al.* 2005). In that study another substance indicative of oxidative stress, ethane, was studied. Ethane concentration was increased significantly after the environmental challenge suggesting that environmental challenge was appropriate to cause oxidative stress in the airways. It is possible that H_2O_2 is a less sensitive biomarker of airway oxidative stress and inflammation, requiring greater antigen stimulation than ethane for increased production in the airways. However other factors may be involved in the regulation of H_2O_2 production in EBC in horses. Further studies are required to assess the usefulness of EBC H_2O_2 for the detection and monitoring of LAI in horses. Furthermore, flow dependency of H_2O_2 production is recognised in humans, however the extent of variability induced by expiratory flows is unknown in horses.

The pH of EBC collected from horses has not been reported previously. In human medicine, pH is considered a robust indicator of LAI (Vaughan *et al.* 2003) despite some methodological issues related to its measurement (Kullmann *et al.* 2007). Given the ease of collection and measurement, EBC pH could be a useful indicator of LAI in horses if it had similar characteristics of repeatability/reproducibility, to that of human EBC and a similar relationship to the presence and severity of disease in the lower airways.

Aims of the Study

The studies contained in this thesis had three aims:

- 1- Develop a portable and non-invasive device to collect EBC from standing horses. A non-portable device has been described previously (Deaton *et al.* 2004b). A portable device for EBC collection from horses was described by Wyse *et al.* (2005) and the device used in this study is a further evolution of that equipment;
- 2- Assess intra-day and inter-day variations in EBC H₂O₂ concentration and pH;
- 3- Determine the effect of lower airway health on EBC H₂O₂ concentration and pH. Specifically, EBC H₂O₂ concentration and pH in healthy horses on pasture, healthy horses housed in stables and horses with LAI were compared.

CHAPTER 3 – MATERIALS AND METHODS FOR COLLECTION AND ANALYSIS OF EBC FROM HORSES

3.1 Collection of EBC

Equipment for collection of exhaled condensate (EBC) was developed and constructed for the purposes of the study.

3.1.1 Construction of the Face Mask

Exhaled breath condensate was collected from horses by use of a face mask that was connected to a condensation chamber by flexible impermeable plastic tubing (Figure 3.1; see Appendix 3 - Figure 6). The mask was obtained by modifying a plastic horse muzzle. Fibreglass was used to occlude all but two holes of the muzzle; each of the remaining two openings of the muzzle were modified to allow the attachment a one-way valve (Salford non-rebreathing valve 35 mm diameter, Cranlea & Company, Birmingham, UK) (see Appendix 3 - Figure 1, Figure 2 and Figure 5). A rubber ring was placed between the valve and the hole in the mask to achieve an airtight connection (see Appendix 3 - Figure 3). An elastic rubber shroud placed on the rim of the mask provided a seal on the face of the horse (see Appendix 3 - Figure 4). Achievement of an airtight seal when the mask is in place on the muzzle of the horse is essential to obtain the negative pressure in the system necessary to activate the valves, ensuring one-way flow of air through the collection device during respiration (Figure 3.2). In addition, an airtight seal avoids contamination of the EBC sample with ambient air. Each valve had an internal diameter of 35mm and initial testing (data not shown) revealed respiration through the device by the horses did not induce any changes in the respiratory pattern (considered as respiratory effort and respiratory rate).

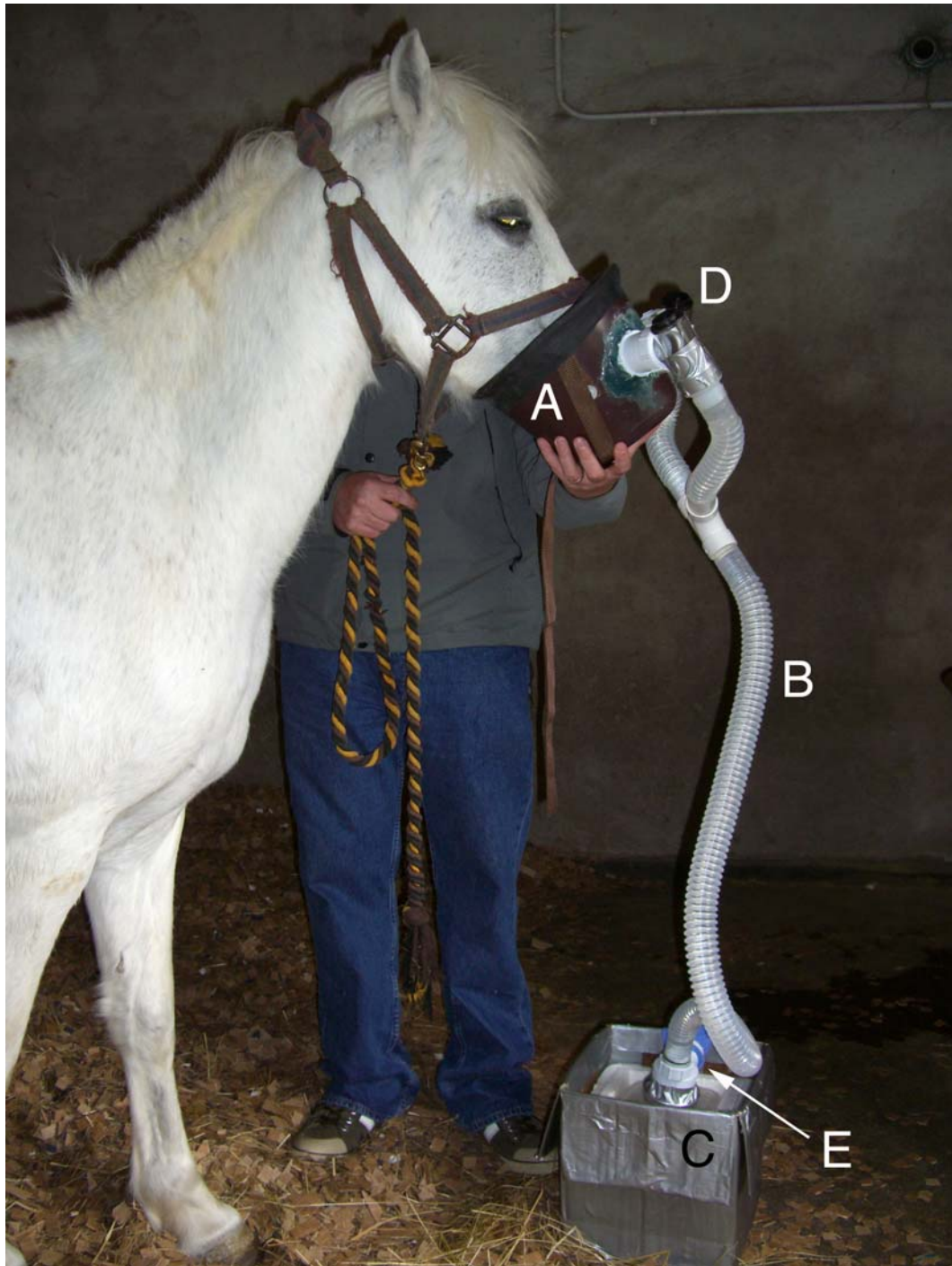


Figure 3.1: Photograph of equipment used to collect exhaled breath condensate from a mature horse. The airtight face mask (A) is attached to flexible rubber tubing (B) that is connected to the condensation chamber immersed in an ethanol/liquid nitrogen slurry (C). The system includes two one-way non-rebreathing valves incorporated into the face mask (D) and a one-way valve at the outlet of the condensation chamber (E) to prevent contamination of the sample with environmental air.

Two valves were used to decrease the resistance of the device to airflow, limiting any change in inspiratory or expiratory flow rates. Avoidance of changes in air flow rates was considered important to prevent any distress to the horse during the procedure.

3.1.2 Condensation Chamber

The one way valves of the mask were connected with flexible rubber/plastic tubes (Cranlea & Company, Birmingham, UK) to the condensation chamber (see appendix Figure 7A). The condensation chamber was comprised of two main parts: an inner polypropylene (PP) beaker in an outer plastic container filled with an ethanol/liquid nitrogen slurry (see Appendix 3 - Figure 7B). The outer chamber containing the slurry was placed in a polystyrene box to improve thermal insulation. The internal shape of the chamber was designed to direct the flow of expired breath towards the bottom of the PP container, similar to that described by Wyse *et al.* (2005). Exhaled breath exited the chamber by flowing up along the outer walls of the condensation chamber and out of the system through a one-way valve (see Appendix 3 - Figure 7A). The rationale to place a third valve in this location was to prevent diffusion of environmental air into the chamber and contamination of the sample. The smaller inner diameter of the condensation chamber was 35mm.

The temperature of the ethanol/liquid nitrogen slurry was monitored with a thermometer with a lower detection limit of -80°C to ensure the temperature was $\leq -80^{\circ}\text{C}$ throughout collection of EBC. Furthermore, the freezing point of ethanol is -114.1°C and some solid ethanol was always observed in the slurry indicating that the temperature of the slurry was very close to that value and indeed $\leq -80^{\circ}\text{C}$. Before collection of each EBC sample, liquid nitrogen was added to the slurry to ensure that similar temperatures were reached for each collection. After the last addition of

liquid nitrogen the temperature was found to remain below -80°C for at least 2 hours (data not shown). Measuring the temperature inside the condensation chamber was not attempted. The temperature on the internal surface of the PP container, where the condensation of breath occurred was considered to be that of the liquid nitrogen/ethanol slurry.

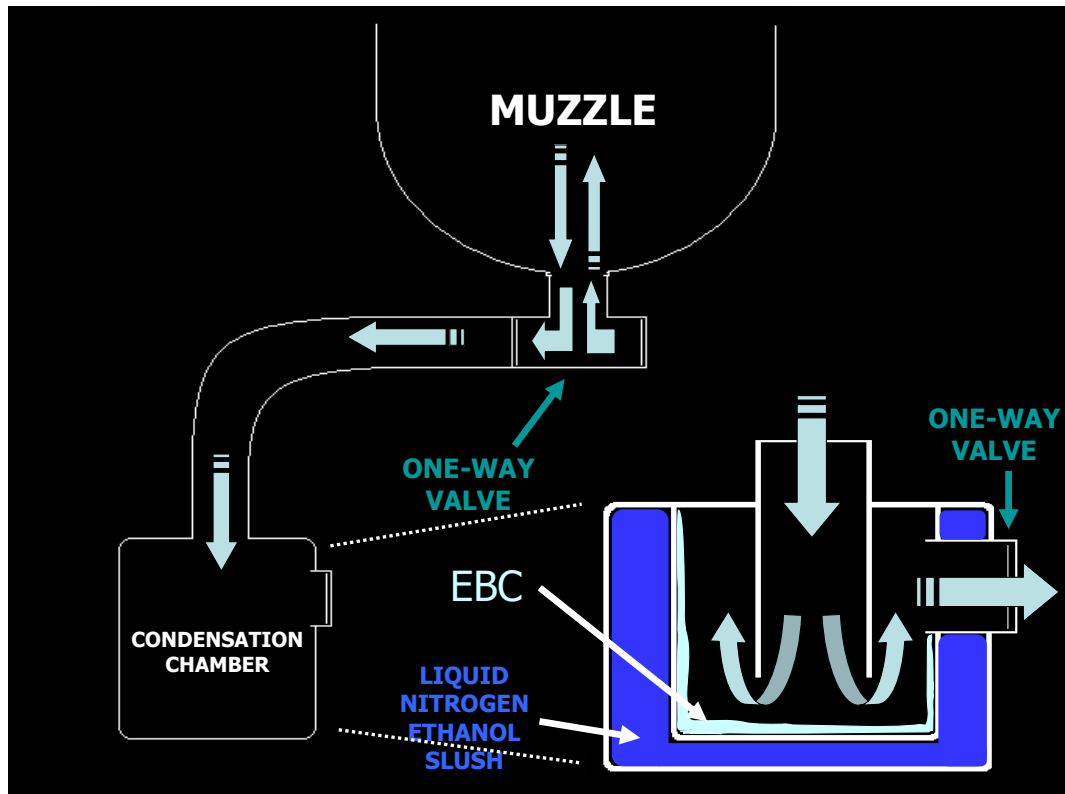


Figure 3.2: Schematic representation of the system used to collect exhaled breath condensate from horses. Exhaled breath is conveyed to the condensation chamber (magnified version on the right) by means of the one-way valve and rubber tubing. The internal shape of the chamber was designed to direct the flow of expired breath towards the bottom of the PP container

3.1.3 Cleaning The Equipment

The masks, valves, connection tubes and PP condensation chambers were cleaned after collection of EBC from each horse. Each part was disconnected and soaked in diluted combination of polymeric biguanide hydrochloride, alkyltrimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride as directed by the manufacturer (Trigene

Advance, Medichem, Kent, UK). After soaking for a period of at least 20 minutes, each piece was rinsed thoroughly with hot tap water and dried at room temperature. This disinfectant was chosen for its broad bactericidal, viricidal, fungicidal and sporicidal properties, including activity against common infectious agents of horses. In addition, the disinfectant solution was compatible with plastics and fibreglass used in the construction of the equipment. The pH of the prepared disinfectant solution is 5.5. The pH measured in a PP container (inner condensation chamber) filled with 1.5ml of tap water was identical before and after disinfection (data not shown) confirming that no disinfectant remained after rinsing of the equipment.

3.1.4 Animals

The signalment and clinical data of the horses used in the studies described in this thesis are provided in the respective chapters describing the individual studies (chapters 3 and 4). Each sample was obtained from standing, non-sedated horses and was achieved in the stall where the horse was housed. This method allowed collection at the owner's premises, without the need to move the horse to the hospital. In addition, some EBC samples were collected from hospitalised patients at the Weipers Centre Equine Hospital Faculty of Veterinary Medicine, University of Glasgow. This possibly decreased the effects of travelling, which could have a potential role in inducing airway inflammation (Ito *et al.* 2001) and influence the results of EBC analysis. However, some horses were collected from immediately after arrival at the hospital, as they had been admitted as an emergency due to exacerbation of RAO or as outpatients presented for reassessment of RAO.

On average, 1.4 ml of EBC was collected in 15 minutes in a frozen solid form (range 0.5 to 7 ml) from each horse. Each sample of EBC obtained

was frozen in a polystyrene container filled with ice and was thawed at room temperature immediately prior to analysis.

3.2 Measurement of EBC H₂O₂ Concentration and pH

3.2.1 Measurement of EBC H₂O₂ Concentration

3.2.1.1 Spectrophotometric assay

Each EBC sample underwent analysis within 1 hour of collection. The concentration of H₂O₂ was determined with a spectrophotometric assay as described by Gallati and Pracht (1985). A solution of horseradish peroxidase was prepared by diluting 44.2mg of powder (see appendix 4 - Figure 1A) in 66ml of distilled water (75IU/L). A solution of 3,3',5,5'-tetramethylbenzidine (TMB) was prepared diluting 1mg of TMB (see appendix 4 - Figure 1B) in 0.5ml of acetone, 0.5ml methanol and 9ml of 0.42M citrate buffer (pH 3.84). Fifteen µl of horseradish peroxidase solution, 140µl of TMB and 140µl of EBC were mixed in quadruplets at room temperature in a darkened room and the reaction stopped after 10 minutes by adding 20µl of 9M sulphuric acid (see Appendix 4 - Figure 2A). The product of this reaction was then immediately analysed with an automated microplate reader (FLUOstar OPTIMA, BMG labtech, UK) at the wavelength for absorbance of 450nm (see Appendix 4 - Figure 2B) (Gallati and Pracht 1985).

The concentration of H₂O₂ was calculated from a standard curve of known concentrations of H₂O₂ prepared for each assay. Linearity of the standard curve was acceptable ($r^2 > 99\%$) for the range 0 to 10µM.

3.2.1.2 Lower detection limit and limit of quantification

The lower detection limit (LDL) was obtained by first drawing a standard curve of H₂O₂ concentration and then calculating the concentration of a 6 series of quadruplicates of blank samples. The LDL was calculated as the mean value of all the blank samples plus three times the standard deviation. The limit of quantification (LOQ) was calculated as three times greater than the LDL. The calculated LDL was 0.1µM and the LOQ was 0.3µM.

3.2.2 Measurement of EBC pH

3.2.2.1 Introduction

As described previously in studies of pH in EBC from human patients (Kullmann *et al.* 2007), several technical issues related to measurement of equine EBC pH need to be addressed and resolved to improve the understanding of this parameter for the assessment of LAI. A principal issue is the time necessary to obtain a stable reading from an EBC sample. Once the probe of a pH meter is placed in a sample of EBC, up to 20 minutes may be necessary to obtain a stable reading of pH. In human studies the reason for the instability of EBC pH may be explained by the presence of volatile compounds in EBC and time required for these compounds to reach stability. The mechanisms of this process is not understood entirely (Kullmann *et al.* 2007). To achieve a more reproducible measurement of pH a common procedure is to bubble a noble gas such as argon through the sample of EBC to facilitate the evaporation of volatile substances (Borrill *et al.* 2006; Kullmann *et al.* 2007). Other authors have tried to standardise EBC pH readings by addition of CO₂ to the sample (Kullmann *et al.* 2007).

The aim of the present study was to evaluate the effect of deaeration with argon and CO₂ on the measured pH of EBC collected from horses. Three methodologies (measurement of plain EBC, after deaeration with argon or deaeration with CO₂) to measure pH of EBC collected from horses were evaluated to determine which provides the most reproducible and time effective measurement.

3.2.2.2 Materials and methods

Animals: Four horses (4 mares; 2 Thoroughbred Cross, 1 Warmblood cross, 1 Pony; age 16±4 years) were used for this study. Horses were stabled on paper bedding, fed soaked hay and the stable window was maintained in an open position to facilitate ventilation. One horse was healthy and the remaining three horses had a history of more than one episodes of LAI in the past (RAO, n=1; IAD n=2); however no horse had clinical signs of LAI at the time of the study. One sample of EBC was obtained from each horse. Sample collection was performed over a period of 15 minutes while the horse was in the stable. The respiratory rate and pattern of the horse were monitored during sample collection. Samples were kept frozen and thawed immediately prior to the time of pH measurement.

pH measurement: measurements of pH in EBC were obtained using a bench pHmeter (pH21, Hanna Instruments Ltd) with an accuracy of ±0.02pH (see Appendix 4 - Figure 3A). A 6mm diameter (see Appendix 4 - Figure 3B), glass body pH-electrode was used to measure the smallest volume samples obtained (at least 0.3ml) (HI-2031B, Hanna Instruments Ltd., UK). The pH meter was calibrated using standard solutions as recommended by the manufacturer before each set of measurements. The measurement of pH was obtained by immersion of the electrode in the sample of EBC and allowing the reading to stabilise (up to 15 min). In

the current study, a measurement of the pH was considered stable when the displayed value was unchanged for a minimum of 3 minutes.

Each sample of EBC collected was divided in 3 aliquots and each pair was assigned to one of the following groups:

1. PLAIN group: the pH was measured in the aliquot of EBC that had not been pre-treated. The numerical value provided by the pH meter was recorded approximately every two minutes (1,3,5,7 and 10 min) and one final reading after the pH of the sample was stable (did not change within 3 minutes).
2. ARGON group: the pH was measured prior to bubbling Argon gas through the sample. The pH was then measured while argon was bubbled through the sample and the measured value recorded approximately every two minutes (1,3,5,7 and 10 min). After 10 minutes the bubbling of Argon was discontinued and pH recorded approximately every two minutes for 10 minutes and one final reading once the measured pH was stable (unchanged for 3 minutes).
3. CO₂ group: the pH was measured prior to bubbling CO₂ through the sample. The pH was then measured while CO₂ was bubbled through the sample and the value was recorded approximately every two minutes (1,3,5,7 and 10 min). After ten minutes the bubbling with CO₂ was discontinued and the value of pH recorded approximately every two minutes for 10 minutes and one final reading once the measured pH was stable (unchanged for 3 minutes).

Statistical analysis: mean, standard deviation (SD) and coefficients of variation (CV) were calculated for these measurements.

3.2.2.3 Results

A mean EBC volume of 3.5 ± 1.1 ml was obtained during the 15 minute collection period. All the horses tolerated the collection procedure and the respiratory rate (10 ± 2 breaths/minute) and respiratory pattern were unaltered during the procedure. The pH measurements obtained for each group are provided in Table 3.1. The mean standard deviation and coefficient of variation for each group are presented in Table 3.2. The pH of EBC samples over time without deaeration, argon deaeration or CO₂ standardization is demonstrated in Figure 3.3.

Table 3.1 : Values of pH in plain aliquots of EBC, aliquots of EBC deaerated with Argon and CO₂ in four horses.

		PLAIN	ARGON		CO ₂	
			10min deaeration	10 min after deaeration	10 min deaeration	10 min after deaeration
Horse 1	mean	5.15	5.325	5.16	4.25	4.065
	SD	0.014	0.007	0.042	0.085	0.007
	CV	0.003	0.001	0.008	0.02	0.002
Horse 2	mean	5.11	5.29	5.08	4.14	4.065
	SD	0.042	0.014	0.057	0.057	0.021
	CV	0.008	0.003	0.011	0.014	0.005
Horse 3	mean	5.76	6.84	6.06	4.23	4.08
	SD	0.014	0.071	0.014	0.014	0.057
	CV	0.002	0.01	0.002	0.003	0.014
Horse 4	mean	5.505	7.215	6.285	4.205	4.07
	SD	0.092	0.064	0.092	0.007	0.014
	CV	0.017	0.009	0.015	0.002	0.003

Table 3.2: Mean, standard deviation (SD) and coefficient of variation (CV) values for measurement of pH in plain aliquots of EBC and aliquots of EBC deaerated with argon or CO₂

	Mean	SD	CV
plain	5.38	0.309	0.057
Argon	5.65	0.615	0.109
CO ₂	4.07	0.007	0.002

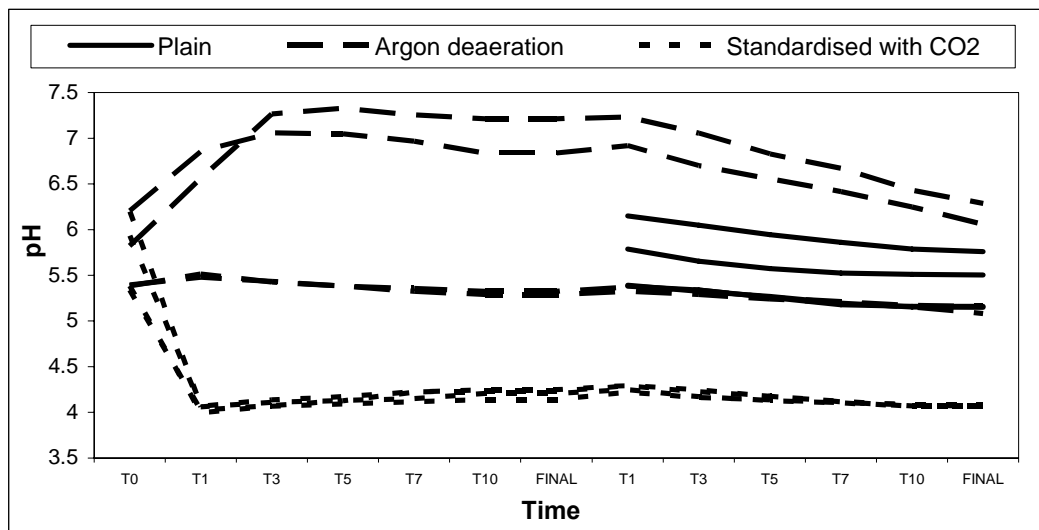


Figure 3.3: pH of exhaled breath condensate over time in plain samples, after Argon deaeration and CO₂ standardisation. The pH was measured at each time point (1,3,5,7 and 10 minutes) during deaeration and when the pH stabilised ("final") after discontinuation of treatment with Argon or CO₂. For the plain EBC samples, pH was measured every 2-3 minutes after until a stable reading was obtained.

3.2.2.4 Discussion

This study suggests that measurement of pH in a plain sample of EBC is more repeatable over time than that of samples that have undergone deaeration with either argon or CO₂ gas. In human studies measurement of EBC pH in plain samples is time-consuming and poorly repeatable and deaeration of the samples with argon has been shown to improve measurement repeatability (Hunt *et al.* 2000; Vaughan *et al.* 2003; Borrill *et al.* 2005). Deaeration of EBC samples by argon deaeration is commonly performed to remove CO₂ as this is an unwelcome "noise" (Vaughan *et al.* 2003; Borrill *et al.* 2005). It was found in studies of human patients that deaeration with argon may have very little effect on samples with pH <6 (Gessner *et al.* 2003; Vaughan *et al.* 2003). Gessner *et al.* (2003) suggested that at pH<6 either CO₂ is not efficiently removed or the pH is mainly determined by other acids (Gessner *et al.* 2003). However, acidic EBC pH could be caused by airway acidification at any level of the

respiratory tract and the source and nature of EBC acidification remain controversial (Effros *et al.* 2006; Hunt 2006). In the current study the effect of Argon deaeration on the pH of EBC collected from horses was unpredictable. The pH increased one pH unit during deaeration, however pH decreased rapidly after discontinuation of deaeration in samples from two animals, while in the samples from two other horses, bubbling argon through the sample had no effect on the pH. Furthermore, after discontinuation of deaeration with argon, the pH of the EBC samples tended to decrease. The reason for the different response to deaeration with argon between samples collected from human and equine patients is unknown. This may reflect a difference in the components of EBC between the two species, but also in the collection methodologies. Further studies would be required to identify and quantify other components of equine EBC. The importance of CO₂ on pH of EBC collected from humans has been widely discussed (Gessner *et al.* 2003; Vaughan *et al.* 2003; Borrill *et al.* 2005; Horvath *et al.* 2005; Borrill *et al.* 2006; Horvath *et al.* 2006; Kullmann *et al.* 2007). The role of other substances, such as ammonia, in the de-aeration-induced changes in EBC pH are not understood (Wells *et al.* 2005; Effros *et al.* 2006). However, some authors found that ammonia concentration in EBC was not affected by deaeration (Kullmann *et al.* 2007). It is possible that ammonia content in EBC collected from horses is high enough to affect the pH (Whittaker A., personal communication).

In a study on human patients, standardization of CO₂ partial pressure in EBC improved the reproducibility of pH measurement by six times compared to argon deaeration (Kullmann *et al.* 2007). The authors found that systematically measuring the partial pressure of CO₂ in the EBC and correcting the pH obtained by calculating the negative logarithmic correlation between pH and partial pressure of CO₂ of EBC considerably improved the reproducibility of EBC pH measurement (Kullmann *et al.*

2007). Since this methodology keeps in account the amount of CO₂ present in EBC and adding CO₂ to the sample stabilises partial pressure of CO₂ in EBC and is more reproducible measure of EBC pH; it should be considered a more reliable method to measure EBC pH than argon deaeration (Kullmann *et al.* 2007). In the current study measurement of partial pressure of CO₂ in EBC samples collected from horses was attempted unsuccessfully in a preliminary study using a portable bloodgas analyser (data not shown). It is possible that the use of a more sensitive blood-gas analyser may give results comparable to the study of Kullmann *et al.* (2007) and justify the use of this methodology in the horse, but this equipment is very expensive and was not available for this study. In the current study, bubbling CO₂ through EBC samples induced a rapid decrease in pH that tended to remain low after CO₂ bubbling was discontinued. The low coefficient of variation between the samples from different animals (CV=0.02) indicates that bubbling CO₂ induced a dramatic change in the pH that is independent of the initial EBC pH as the procedure saturates the sample of EBC with CO₂, which in solution is in equilibrium with carbonic acid (and H⁺ ions).

To the author's knowledge there is one report in the literature of measurement of EBC pH from calves (Zollinger *et al.* 2006; Knobloch *et al.* 2008). Zollinger *et al.* (2006) found that the pH was slightly acidic in these species compared to humans, ranging from 5.43 to 6.46 (mean 5.74 ± 0.21) in non-degassed EBC samples. Comparing these results with those from other species may give more information to explain the different response to deaeration between human and equine EBC. The results of this study show that pH measurement is most reproducible on the plain sample compared the argon and CO₂ standardisations.

3.3 Repeatability of the Methodology for Collection and Analysis of EBC H₂O₂ Concentration and pH

3.3.1 Introduction

The method for collection and analysis of EBC H₂O₂ concentration and pH to determine repeatability and magnitude of any experimental errors has never been assessed in the horse. Many factors contribute to variability of EBC H₂O₂ concentration and pH measurements reported in studies from human patients (Olin *et al.* 2006). Studies in human medicine have assessed variability of EBC H₂O₂ concentration (Nowak *et al.* 2001; van Beurden *et al.* 2002a) and pH (Vaughan *et al.* 2003; Borrill *et al.* 2005; Bloemen *et al.* 2006) using methods of collection and analysis widely validated (Vaughan *et al.* 2003; Goldoni *et al.* 2005; Soyer *et al.* 2006). Among the factors that contribute to the variability of the methodology is that H₂O₂ is a very reactive molecule that can undergo rapid degradation during interaction with different materials (Horvath *et al.* 2005). Samples of EBC must be analysed immediately or frozen and stored at temperatures $\leq -70^{\circ}\text{C}$ until analysis of H₂O₂ concentration (Horvath *et al.* 2005). The laboratory method used for determination of H₂O₂ concentration in EBC can influence the results as the physiological concentrations of EBC H₂O₂ in human patients are often very close to or even less than, the lower detection limit of many of these assays (about 0.1 $\mu\text{m/L}$) (Gallati and Pracht 1985; van Beurden *et al.* 2002b; Svensson *et al.* 2004). The length of EBC collection time has been studied as a possible cause for variability in H₂O₂ concentration, however no significant difference in EBC H₂O₂ concentration was found when samples obtained over a collection period of 4 or 10 minutes from human patients were compared (Svensson *et al.* 2004). To date none of the published equine

studies (Deaton *et al.* 2004b; Wyse *et al.* 2005) assessed intra-day and inter-day variations, or whether the length of collection time can have an effect on H₂O₂ concentration. Different methods of sample collection, sample storage and analysis could contribute substantially to variations in measured values of parameters of interest. Therefore, it is important to quantify the variability in specific parameters with methods of collection and analysis to allow a more objective interpretation of any study using the methodology, to understand to what extent the variability between repeated measurements is reflective of a true physiological variation and of experimental errors.

The aim of this study is to evaluate the repeatability of methods of collection and analysis of EBC using the methodology described in paragraphs 3.1 and 3.2 in a group of horses.

3.3.2 Materials and Methods

Exhaled breath condensate was collected from 4 horses (4 mares; 2 Thoroughbred Cross, 1 Warmblood cross, 1 Pony; age 16±4 years). One horse was healthy and the remaining three horses had a history of RAO (n=1) or IAD (n=2). No horse had clinical signs of LAI at the time of the study. Horses were stabled on paper bedding and fed soaked hay and the stable's window was maintained in an open position to increase stable ventilation.

Samples of EBC were collected from horses in the stable at different times of the day (4 samples in the morning and 2 samples in the afternoon). Exhaled breath condensate was obtained from two horses on two separate occasions and from two horses once. At each sample collection time point two samples of EBC were collected with an intervening period of 5 minutes. Each sample was analysed for determination of H₂O₂ and pH

measurement as described in paragraphs 3.1 and 3.2. Collection time was 10 minutes for each sample and the samples were stored in ice and analysed within one hour from collection. Hydrogen peroxide was determined using the spectrophotometric assay described by Gallati and Pracht (1985). The pH was measured from plain samples using a bench pH meter.

The coefficient of variation was calculated for each pair of H₂O₂ and pH measurements from each horse.

3.3.3 Results

Six paired measurements were obtained resulting in a total of 12 samples of EBC. The results for each horse are summarised in Table 3.3.

All the horses tolerated the collection procedure. The mean respiratory rate during collection of EBC was 10±1 breaths per minute and the mean volume of EBC retrieved was 1.1±0.7ml per sample. The calculated mean coefficients of variation for each paired measurement were 0.05 and 0.01 for H₂O₂ and pH, respectively. For the sample collected in the morning in horse 2 not enough EBC was collected in 5 minutes and H₂O₂ concentration was measured only.

Table 3.3: Results of the study of the repeatability of the collection and analysis methodology for EBC H₂O₂ and pH. Exhaled breath condensate pH, H₂O₂ concentration, respiratory rate and volume collected for each sample and the coefficients of variation for pH and H₂O₂ repeated measurements are reported. For horse 2 am not enough sample was available and H₂O₂ concentration was measured only.

EBC	Round	pH	H ₂ O ₂ (μ M)	Resp Rate (Bpm)	Volume (ml/10min)		H ₂ O ₂ (μ M)	pH
Horse 1 Am	1	5.67	3.48	12	2.5	AVG	3.6	5.74
	2	5.8	3.72	12	2.5	SD	0.17	0.09
						CV	0.05	0.02
Horse 2 Am	1	-	5.67	12	0.6	AVG	5.46	-
	2	-	5.24	10	0.5	SD	0.3	-
						CV	0.06	-
Horse 1 Am	1	6.01	6.7	10	0.8	AVG	6.76	5.99
	2	5.97	6.81	11	0.9	SD	0.08	0.03
						CV	0.01	0
Horse 2 Am	1	5.81	3.08	8	0.8	AVG	3.2	5.77
	2	5.72	3.31	9	0.7	SD	0.16	0.06
						CV	0.05	0.01
Horse 3 Pm	1	5.43	1.99	10	1.2	AVG	2.09	5.48
	2	5.52	2.18	11	1.1	SD	0.13	0.06
						CV	0.06	0.01
Horse 4 Pm	1	5.25	0.74	9	0.7	AVG	0.71	5.28
	2	5.31	0.67	9	0.8	SD	0.05	0.04
						CV	0.07	0.01
			AVG	10.3	1.09	Tot CV	0.05	0.01
			SD	1.36	0.69			

3.3.4 Discussion

None of the previously published studies of EBC H₂O₂ in horses focused on the assessment of the methodology of collection and analysis to achieve an estimation of the experimental error (Deaton *et al.* 2004b; Wyse *et al.* 2005). No data are published on the assessment of the repeatability of the measurements reported in these studies and this may influence the reliability of the results reported. In the study by Deaton *et al.* (2004a) there was a significant difference in EBC H₂O₂ concentration during acute and remission phase in horses with RAO. Since data on the repeatability of

the collection and analysis methods are not reported in that study it is unknown to what extent the methodological error influenced these results. However the detection of a highly significant statistical difference in EBC H_2O_2 concentration between acute RAO and RAO in remission, suggests that the methodology used in that study is reliable. In the study by Wyse *et al.* (2005) there was no significant difference in EBC H_2O_2 concentration in horses with RAO before and after environmental challenge. Several limitations, such as small number of horses and failure to induce remarkable LAI, were discussed by the authors, but no comment was made on the methodology of collection (Wyse *et al.* 2005). The repeatability of collection and analysis methods has been widely evaluated in studies on EBC H_2O_2 concentration and pH from human patients (Nowak *et al.* 2001; van Beurden *et al.* 2002a; Vaughan *et al.* 2003; Borrill *et al.* 2005; Bloemen *et al.* 2006). The recommendation in human medicine is to report data on the assessment of methodology in a study unless the methodology used is validated elsewhere (Horvath *et al.* 2005). The results of this study indicate that measurement of the pH in EBC samples is more repeatable (CV: 0.01) than that of H_2O_2 concentration (CV: 0.05). These findings indicate that while some variation in values of H_2O_2 concentration and to a lesser degree EBC pH may be obtained when collecting and analysing EBC using the described methodology, the variation is very small. Knowing the magnitude of the variation of the specific methodology used is important as it allows a more objective interpretation of the data obtained. Variability of H_2O_2 concentration and pH in EBC may result from cumulative differences in the steps of the analytical process. Slight differences in temperature of the condensation chamber, respiratory rate, length of sample collection, time between collection and analysis (the first sample collected waits longer to be analysed) in room temperature and relative humidity are all possible contributors. Even though each factor may contribute minimally to the variability, influences may become appreciable in a cumulative manner.

Differences in temperature of the collection chamber have been reported to influence the concentration of H₂O₂ in studies from human patients (Goldoni *et al.* 2005). In addition respiratory flow rates have been shown to influence EBC H₂O₂ concentration in studies from human patients (Schleiss *et al.* 2000). Previous studies reported collection of EBC at tidal breathing in non-sedated horses but the exact flow rates were not reported (Deaton *et al.* 2004b; Wyse *et al.* 2005). In the present study, EBC collection was also performed at tidal breathing, however the flow rates were not measured and it is therefore unknown to what extent this may have biased the results. Measurement of flow rates in the standing non-sedated horse requires equipment that was not available for this study. All horses were accustomed to and tolerated the collection procedure therefore it was assumed that there was no change in flow rates thorough out the study. Insufficient EBC sample volume was retrieved for both analysis of H₂O₂ and pH measurement and measurement of H₂O₂ concentration was performed only. Collection time, time to analysis and storage temperature of the samples significantly affect EBC H₂O₂ concentration (Olin *et al.* 2006). Sampling for longer than 15 minutes and storing samples at -20°C, or leaving the melted sample unattended for longer than 1 hour can cause a significant decrease in H₂O₂ concentration (Horvath *et al.* 2005; Olin *et al.* 2006). To comply with the recommendations of ATS/ERS Task Force on Exhaled Breath Condensate, in the current study collection time was 10 minutes and analysis was performed within one hour from collection with the sample stored frozen at <-80°C and melted only at the time of analysis.

Measurement of pH of EBC after argon deaeration has been shown to have excellent repeatability in humans (Vaughan *et al.* 2003). No studies have been reported on the repeatability of pH measurement of EBC in horses. The findings of the current study indicate that quantification of EBC pH is highly repeatable.

This study had some limitations. A small number of horses were available for the study and two horses were sampled twice to increase the number of data available. The type of horse used was also heterogeneous and it is unknown if using horses of many different breeds may significantly affect the results. The samples of EBC collected were stored frozen in ice and analysed within one hour from collection. Some difference between the measurements for each pair of samples obtained could have been due to the difference in time between collection of the samples (~15 minutes). However these limitations were unavoidable with the design of the current study and are unlikely to have significantly biased the results. Whether using a device to collect two samples at once would have reduced the CV is unknown. In one pony insufficient volume of EBC was collected to perform both H_2O_2 concentration and pH measurements. It is possible the smaller volume collected may have been a reflection of a smaller tidal volume in this breed. Using similar but smaller equipment may maximize collection of EBC from smaller breeds.

The CV obtained for repeated collection and analysis of EBC H_2O_2 concentration and pH were considered acceptable for the application of the methodology for future studies of healthy horses and horses with lower airway disease.

CHAPTER 4 – STUDY OF THE INTRA-DAY AND INTER-DAY VARIABILITY IN EBC H₂O₂ CONCENTRATION AND pH

4.1 Introduction

Considerable variation in EBC H₂O₂ concentration has been described in human studies; an intra-day CV of over 40% in both healthy non-smoking humans and patients with asthma was reported by van Beurden *et al.* (2002). Despite this variability, a correlation between presence and severity of disease and EBC H₂O₂ concentration in humans has been reported (van Beurden *et al.* 2002a). These authors suggested this finding was consequent to the mean EBC H₂O₂ concentration in the population being relatively stable overtime. In contrast, EBC pH values in healthy humans have been found to be very stable overtime, with mean intraweek and intraday coefficients of variation of 4.5% and 3.5% respectively reported by Vaughan *et al.* (2003). Good correlation between degree of lower airway acidification and presence and severity of lower airway inflammation in humans has been reported (Hunt *et al.* 2000).

The assessment of inter-day and intra-day variability in both EBC H₂O₂ concentration and pH is essential for the validation of the use of these parameters as indicators of respiratory health/disease in horses. Variability of EBC H₂O₂ concentration measurements from horses after environmental challenge has been described by Deaton *et al.* (2005). In that study the variability reported was thought to be due to either physiological variation or a response of the airways to inhaled components of the air in the environment of the stable (Deaton *et al.* 2005a). In that study there was an increase in EBC H₂O₂ concentration in horses with RAO after environmental challenge. However, there were also changes in EBC H₂O₂ concentration over a period of two weeks in both healthy and RAO horses following environmental challenge. The authors concluded that this

variability was the summation of the response to the challenge, experimental errors and inter-day physiological variation in EBC H₂O₂ concentration (Deaton *et al.* 2005a).

Exhaled breath condensate pH has not been studied previously in the horse.

The aims of this study were to:

- 1- assess the intra-day and inter-day variability of EBC H₂O₂ concentration and pH in healthy horses and in horses with LAI;
- 2- determine whether EBC H₂O₂ concentration and pH were associated with the health status of the respiratory tract (i.e. were there differences in these parameters between healthy horses and horses with LAI).

4.2 Materials and Methods

4.2.1 Animals

Sixteen horses were used in the study and were divided into two groups. A control group comprised of eleven healthy horses free of any history or clinical signs of respiratory disease (4 mares, 7 geldings; 6 Cobs, 2 Ponies, 2 Warmblood, 1 Thoroughbred cross; mean age: 12 years, ranging from 5 to 20 years). Clinical examination findings of the control group horses, including auscultation during forced rebreathing, were within normal limits. Eight of the control horses lived at the same livery yard and each was stabled on wood shavings in a traditional stall and fed dry hay. The stable ceiling communicated between stalls and one window was opened at all times in each stall to provide ventilation. The remaining 3 horses of the control group were hospitalised at the Weipers Centre Equine Hospital, Faculty of Veterinary Medicine, University of Glasgow. These

horses were stabled on paper bedding in stalls with good ventilation and were fed soaked hay. None of the control group horses underwent anaesthesia or received any medication for at least 72 hours prior to or during the period of the study.

The second group included five mares with lower airway inflammation (LAI). The LAI group was comprised of 2 Thoroughbred cross mares, 2 Warmblood cross mares and 1 Pony mare. The mean age of the LAI horses was 16 years (range 6 to 24 years). Lower airway inflammation was confirmed by history, clinical signs (exercise intolerance, occasional cough and serous nasal discharge) and BALF cytology (neutrophils > 5%). The clinical examination and BALF cytology findings for each horse with LAI are summarised in table 4.1.

Table 4.1: Clinical and bronchoalveolar lavage cytology data from 5 horses with lower airway inflammation

	Signalment	Clinical signs	BAL cytology	
Horse 1	15 y.o. TB X Mare	Occasional nasal discharge occasional cough	Neutrophils	18%
			Macrophages	23%
			Lymphocytes	54%
			Mast cells	5%
Horse 2	22 y.o. TB X Mare	Occasional cough	Neutrophils	13%
			Macrophages	41%
			Lymphocytes	35%
			Mast cells	11%
Horse 3	24 y.o. Pony Mare	Occasional cough, occasional nasal discharge	Neutrophils	11%
			Macrophages	33%
			Lymphocytes	45%
			Mast cells	11%
Horse 4	15 y.o. WB X Mare	Exercise intolerance prolonged recovery time from exercise occasional cough	Neutrophils	15%
			Macrophages	30%
			Lymphocytes	50%
			Mast cells	5%
Horse 5	6 y.o. WB X Mare	Cough increased inspiratory and expiratory effort bilateral nasal discharge	Neutrophils	64%
			Macrophages	12%
			Lymphocytes	22%
			Mast cells	2%

TB= thoroughbred, WB= Warmblood, X=cross, y.o=years old

The horses in the LAI group were stabled in stalls with good ventilation on paper bedding and fed soaked hay. None of the horses had received any medication in the previous 4 months.

4.2.2 Collection of EBC Samples and Analysis

The EBC samples were collected using the methodology described in paragraph 3.1. The collection time for each sample was 10 minutes. Each sample of EBC was stored on ice immediately after collection and thawed immediately prior to analysis. Hydrogen peroxide and pH were measured within one hour from collection as described in paragraph 3.2.

4.2.3 Study design

Intra-day variability of both EBC H₂O₂ concentration and pH was assessed by collection of EBC samples at 6 hourly intervals over a 12 hour period (6am, 12pm, 6pm) from each horse of both groups.

To determine inter-day variability, a single EBC sample was obtained from each horse of both the control and LAI groups at 12pm daily for three consecutive days.

4.2.4 Statistical analysis

The mean coefficient of variation (CV) of intra-day and inter-day measurements of both H₂O₂ and pH were calculated for each of the control and LAI groups. For each group, the intra-day CV was calculated as the mean of the intra-day CV of each horse for both H₂O₂ and pH measurements. For each group the CV of inter-day measurements of H₂O₂ and pH was obtained as the mean of the inter-day CV values for each of the horses in each group. Mean and standard deviation (SD) of H₂O₂

concentration and pH of both the control and LAI groups were calculated from all of the measurements for each group.

A one-way ANOVA was used to detect significant differences of EBC H₂O₂ concentration for intra-day variability and inter-day variability in both the control and LAI groups. Differences in both intra-day and inter-day EBC pH and differences in EBC H₂O₂ concentration and pH between control and LAI groups were assessed using the Mann-Whitney test. Relationship between EBC H₂O₂ concentration and pH was studied in both groups using the Pearson's correlation rank. Significance was set at $p < 0.05$.

All studies were approved by the Animal Ethics and Welfare Committee of the Faculty of Veterinary Medicine, University of Glasgow.

4.3 Results

A total of 80 samples of EBC were collected from the 16 horses involved in the study. Hydrogen peroxide concentration was measured in all samples. pH was measured in 79 samples; measurement was not possible in one sample because insufficient EBC volume was retrieved. All horses tolerated the procedure of EBC collection. For the control group, the mean volume of EBC collected was 2.7 ± 1.4 ml and mean respiratory rate during collection was 10 ± 3 bpm. There was a tendency for a decrease in the respiratory rate with repeated collections. For the LAI group, the mean volume of EBC collected was 2.3 ± 1.0 ml and mean respiratory rate during collection was 12 ± 4 bpm. No significant differences were present between the two groups (in respiratory rates during collection or volume of EBC collected). The mean H₂O₂ concentration in the control group was 2.3 ± 1.8 μ M and 2.6 ± 3.9 μ M in the LAI group. The mean pH in the control group was 6.00 ± 0.61 and 5.77 ± 0.26 in the LAI group. (Table 4.2). Data for each horse in each group are reported in Table 4.3 and in Table 4.4.

Table 4.2: Mean values (\pm SD) of all the measurements of H_2O_2 concentration and pH from the control and LAI groups

	Control group		LAI group	
	Mean	SD	Mean	SD
H_2O_2 (μM)	2.3	1.8	2.6	3.9
pH	6.00	0.61	5.77	0.26

Table 4.3: Coefficient of variation values for intra-day and inter-day measurements of both H_2O_2 concentration and pH in exhaled breath condensate from 11 horses without evidence of lower airway inflammation (control group).

	Intra-day		Inter-day	
	H_2O_2	pH	H_2O_2	pH
Horse 1	1.232	0.039	1.288	0.042
Horse 2	0.330	0.004	0.573	0.007
Horse 3	0.086	0.074	0.074	0.027
Horse 4	0.505	0.072	0.932	0.072
Horse 5	1.406	0.058	0.699	0.110
Horse 6	0.391	0.042	0.697	0.085
Horse 7	0.965	0.047	0.966	0.107
Horse 8	0.497	0.107	0.552	0.053
Horse 9	0.550	0.064	0.409	0.098
Horse 10	0.997	0.028	0.780	0.061
Horse 11	0.558	0.053	0.093	0.071
Mean	0.683	0.053	0.642	0.067
SD	0.409	0.027	0.364	0.033

Table 4.4: Coefficient of variation values for intra-day and inter-day measurements of H_2O_2 concentration and pH in exhaled breath condensate of the 5 horses in the LAI group.

	Intra-day		Inter-day	
	H_2O_2	pH	H_2O_2	pH
Horse 1	0.243	0.042	1.084	0.004
Horse 2	0.423	0.053	1.267	0.038
Horse 3	1.732	0.026	1.100	0.047
Horse 4	0.151	0.007	1.106	0.049
Horse 5	0.458	0.058	0.597	0.055
Mean	0.601	0.037	1.031	0.039
SD	0.645	0.021	0.254	0.020

There was no significant difference in EBC H₂O₂ concentration ($p=0.859$) or pH ($p=0.131$) between the two groups. The calculated CV values for EBC H₂O₂ concentration and pH of the control group are shown in Table 4.3. For the control group the mean CV for H₂O₂ concentration was 0.683 and 0.64 for intra-day and inter-day measurements, respectively. In this group the calculated CV for EBC pH was 0.53 and 0.67 for intra-day and inter-day measurements, respectively.

The calculated CV for the LAI group are shown in Table 4.4. For this group the CV for H₂O₂ concentration was 0.601 and 1.031 for intra-day and inter-day measurements, respectively. For EBC pH, the calculated CV was 0.37 and 0.39 for intra-day and inter-day measurements, respectively.

The mean H₂O₂ concentration in the control group was $2.3 \pm 1.8 \mu\text{M}$ and $2.6 \pm 3.9 \mu\text{M}$ in the LAI group. The mean pH in the control group was 6.00 ± 0.61 and 5.77 ± 0.26 in the LAI group. There was no significant difference in EBC H₂O₂ concentration ($p=0.859$) or pH ($p=0.131$) between the two groups (Table 4.2).

No significant difference in EBC H₂O₂ concentration for intra-day measurements was present in the control group ($p=0.132$) (Figure 4.1) or LAI group ($p=0.475$) (Figure 4.2). No significant inter-day difference in H₂O₂ concentration was present in the control group ($p=0.088$; Figure 4.1). A significant difference in the inter-day repeated measurements of H₂O₂ concentration was present in the LAI group ($p=0.019$) (Figure 4.2).

No significant difference in intra-day EBC pH measurements was present in the control group ($p=0.572$) (Figure 4.3; Table 4.5) or LAI group ($p=0.811$) (Table 4.6). No significant difference in inter-day EBC pH measurements was present in the control group ($p=0.299$) (Figure 4.3) or LAI group ($p=0.231$) (Table 4.5). No correlation was present between EBC H₂O₂ concentration and pH in the control group (Pearson's correlation

coefficient=-0.127; p=0.354) (Figure 4.3) or LAI group (Pearson's correlation coefficient=-0.277; p=0.181) (Figure 4.4).

Table 4.5: Intra-day and inter-day EBC H₂O₂ concentration and pH collected on 5 occasions over a 54 hour period from the horses in the control group (n=5) to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30hr and 54 hr).

	H ₂ O ₂ (μM)					pH				
	0h	6h	12h	30h	54h	0h	6h	12h	30h	54h
Horse 1	1.8	1.2	0.3	0.0	0.1	5.84	5.58	5.47	5.90	5.92
Horse 2	1.6	8.8	5.0	5.3	3.1	4.96	5.12	5.12	5.16	5.19
Horse 3	3.2	3.1	3.7	3.6	3.7	5.24	5.69	5.02	5.02	5.28
Horse 4	0.8	1.7	3.8	1.7	0.9	5.38	5.54	6.21	6.36	5.89
Horse 5	1.6	0.6	3.9	0.0	1.0	5.91	6.34	6.32	5.82	7.30
Horse 6	0.7	3.0	1.4	1.9	2.9	6.22	6.41	6.97	6.66	7.36
Horse 7	0.4	0.6	1.1	0.0	3.5	5.89	5.78	5.82	6.28	7.01
Horse 8	1.8	5.2	3.6	1.7	6.0	6.87	6.29	6.93	5.59	6.29
Horse 9	1.4	2.6	2.5	0.7	1.2	5.49	5.46	6.20	5.77	6.69
Horse 10	2.0	1.6	5.1	0.5	1.1	6.86	6.28	6.36	6.03	6.09
Horse 11	2.5	4.0	2.6	1.1	3.0	5.79	6.38	6.36	5.80	6.67
Mean	1.6	2.9	3.00	1.50	2.4	5.86	5.90	6.07	5.85	6.34
SD	0.8	2.4	1.58	1.66	1.7	0.61	0.46	0.65	0.49	0.74

Table 4.6: Intra-day and inter-day EBC H₂O₂ concentration and pH collected on 5 occasions over a 54 hour period from the horses in the LAI group (n=5) to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30hr and 54 hr). *= statistically significant (p=0.019)

	H ₂ O ₂ (μM)					pH				
	0h	6h	12h	30h	54h	0h	6h	12h	30h	54h
Horse 1	1.6	1.6	2.4	0.0	4.1	5.52	5.66	5.99	5.70	5.66
Horse 2	1.9	2.1	3.9	1.2	14.9	5.38	5.61	5.97	6.05	5.80
Horse 3	0.0	0.5	0.0	0.7	3.8	5.75	6.03	5.79	6.09	5.58
Horse 4	1.7	1.6	3.6	0.0	4.3	5.99	6.07	5.43	6.29	5.71
Horse 5	1.7	1.3	4.6	1.1	4.8	5.87	6.05	5.25	5.82	5.22
Mean	1.37	1.87	2.44	0.60	6.38*	5.70	5.76	5.81	5.99	5.59
SD	0.77	1.11	1.88	0.57	0.57	0.25	0.28	0.33	0.22	0.23

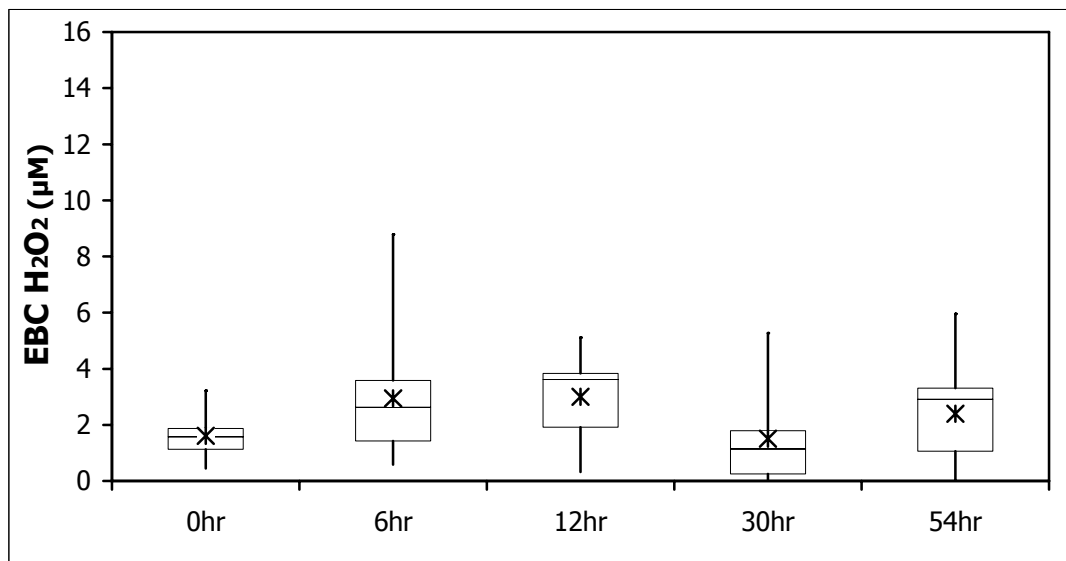


Figure 4.1: Box and whiskers quartile chart of H₂O₂ concentration in EBC of control horses (n=11) collected on 5 occasions over a 54 hour period to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30hr and 54 hr); *=mean

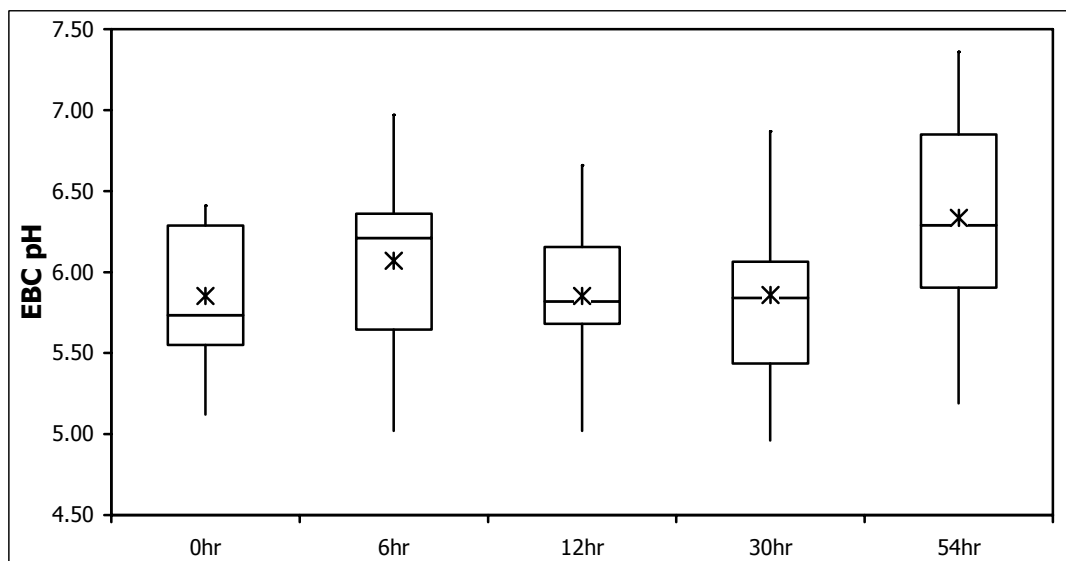


Figure 4.2: Box and whiskers quartile chart of the pH of EBC of control horses (n=11) collected on 5 occasions over a 54 hour period to assess intra-day variability (0hr, 6hr and 12 hr) and inter-day variability (6hr, 30 hr and 54 hr). No significant difference was present (p=0.299); *=mean.

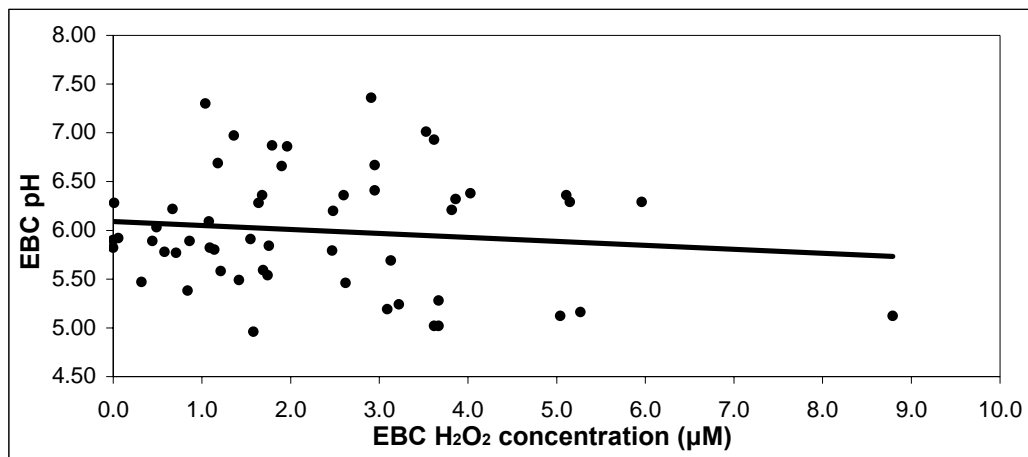


Figure 4.3: Lack of correlation between EBC H_2O_2 concentration and pH in the horses of the control group (Pearson's correlation coefficient=-0.127; $p=0.354$)

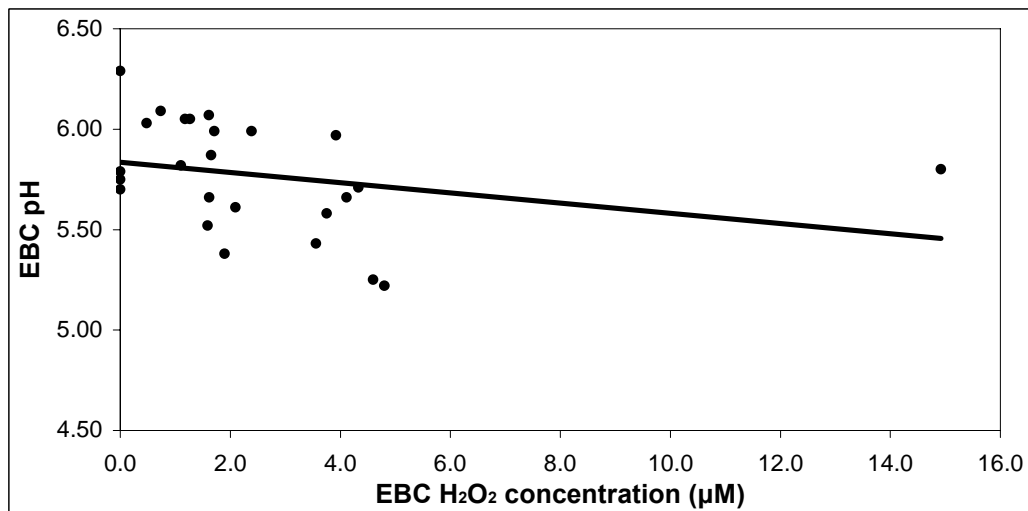


Figure 4.4: Lack of correlation between EBC H_2O_2 concentration and pH in the horses of the LAI group (Pearson's correlation coefficient=-0.277; $p=0.181$)

4.4 Discussion

The results of this study demonstrate that within and between days there is a considerable variation in H_2O_2 concentration in EBC in both healthy horses and horses with LAI. The mean coefficient of variation in the LAI group was approximately double that of the control group. However, this difference was not statistically significant, probably due to the high variability present between horses in both groups. The variability in EBC H_2O_2 concentration observed in this study may partially explain the lack of

a significant increase of EBC H_2O_2 concentration after environmental challenge described in previous studies (Deaton *et al.* 2005a; Wyse *et al.* 2005). Results of the intra-day and inter-day variability studies suggest that EBC H_2O_2 concentration is subject to wide fluctuations in individual horses. The use of one-way ANOVA identified a significant difference in the inter-day variation of H_2O_2 concentration in the five horses with LAI. Examination of the data suggest that the concentration of H_2O_2 was significantly greater on day 3 compared to days 1 and 2. There were no management changes during the period of the study that were likely to have exacerbated airway inflammation and the difference in EBC H_2O_2 concentration is most probably consequence of the wide variability for repeated measurements and small number of horses in this group. Conversely, the inter-day variation was not significant in the control group and may reflect the larger number of horses in the control group in comparison to the LAI group. Inter-day variability of H_2O_2 concentration in the control group and LAI group could be due to effects of low grade/subclinical airway inflammation and/or different sensitivities of cells in the airways to environmental irritants or antigens. The number of neutrophils and other leukocytes that migrate into the airways as part of the physiology of the lower airways and the degree of activation of these cells may vary and influence the concentrations of H_2O_2 in EBC. In addition, the equipment used in this study and any methodological errors could induce some degree of variability in measured H_2O_2 concentration, however these were likely minimal as indicated by the results of the assessment of the methodology in paragraph 3.3. Differences in respiratory rates and flow rates of the horses could also have played a role in EBC H_2O_2 concentration variability. Expiratory flow rates have been shown to influence H_2O_2 concentration in human studies (Schleiss *et al.* 2000). However, the influence of flow rates on EBC H_2O_2 concentration in horses has not been reported. In the current study EBC was collected during tidal breathing in all horses. In addition, it is possible that the

degree of environmental humidity and hydration status of the horses could have influenced results by influencing the dilution of constituents of EBC. The variability of H₂O₂ concentration in EBC in the current study (60-103%) is higher than that reported for healthy humans (42%) and humans with chronic obstructive pulmonary disease (45%) (van Beurden *et al.* 2002a). In humans, variability in EBC H₂O₂ concentration is likely to be dependent on expiratory flow rates and circadian rhythms (Schleiss *et al.* 2000; Nowak *et al.* 2001; van Beurden *et al.* 2002a). Diurnal variation in EBC H₂O₂ concentration has not been studied in the horse and any diurnal effect in the current study is unknown. The large variability in H₂O₂ concentration observed in the current study could explain the lack of significant difference between healthy horses and horses with LAI.

The pH of EBC collected from horses has not been reported previously. The degree of variability in pH both within and between days in the horses of this study was low ($\leq 6.7\%$) suggesting that EBC pH is largely unchanged over time. Similarly, minimal variability in EBC pH has been reported in human studies. In healthy human subjects the variation was 3.5% and 4.5% for intra-day and intra-week repeated measurements, respectively (Vaughan *et al.* 2003). In the current study, some of the variation in EBC pH may have been contributed to by experimental/methodological error. However, as discussed in paragraph 3.3, variability due to methodology was found to be approximately 1% for pH. The magnitude of the CV value for EBC pH in the current study are too high to be attributed only to experimental errors. In the absence of an appreciable change in the management of the horses, the variations in EBC H₂O₂ concentration and pH may represent physiological processes in the lower (and possibly upper) airways.

The mean concentrations of H₂O₂ in EBC were slightly higher in LAI group compared to the control group, however no statistically significant

difference was detected. In addition, the pH of EBC was not significantly different between the two groups, although there was a slight trend for the control group to have a higher pH than the LAI group ($p=0.13$). The neutrophil differential count in the BAL of the LAI group was between 11% and 18% in 4 of the 5 horses of this group suggesting mild inflammation. Only one horse had marked BALF neutrophilia (64%) consistent with marked inflammation. Hydrogen peroxide is produced by activated neutrophils and is possible that activation of airway neutrophils in the horses of the LAI group was limited as they were stabled in a low dust environment. Moreover the clinical signs of the horses in the LAI group were mild. Moderate exercise intolerance and prolonged recovery time after exercise were present in two horses, while the other three horses in this group had increased respiratory effort. Cytological examination of BALF was necessary to confirm the presence of LAI in these horses. It would have been preferable to perform a BAL and cytological analysis of BALF on each of the horses of the control group to exclude the presence of LAI and achieve a more objective differentiation between groups, however this procedure is invasive and it was not appropriate to perform on clinically healthy horses for ethics and welfare reasons. The absence of respiratory disease in the history of each horse and absence of clinical signs of LAI were considered acceptable to identify horses likely free of lower airway disease. It has been shown that examination of historical information can identify horses with RAO (Hotchkiss *et al.* 2006; Ramseyer *et al.* 2007), however this approach has not been assessed for the identification of horses with IAD, and it may be less sensitive in this disease group due to more subtle clinical signs.

It is possible that inclusion of horses with more marked clinical signs of respiratory disease and greater airway inflammation would have produced a greater difference between control and LAI group for both for EBC H_2O_2 concentration and pH. However, the wide variability in EBC H_2O_2

concentration may preclude the accurate identification of individual patients with LAI. It remains possible that quantification of EBC H₂O₂ may be a useful indicator of severity of LAI, as reported previously (Deaton *et al.* 2004b). While EBC pH had minimal variability within and between days, it was not a useful marker of mild LAI. Examination of horses with more marked LAI, for example acute RAO, may help to elucidate whether EBC pH is altered with airway inflammation.

Another confounding factor in this study was that control and LAI groups were housed in two different environments. The management at the yard where the majority of the control horses were housed (8/11) was more traditional (wood shavings, dry hay diet) than that used to house the LAI group horses (paper bedding, good stable ventilation). Although no data on the quality of the air in the environment of the housing on the control horses were available, it is possible that the horses of this study were exposed to greater amounts of inhalatory antigens/allergens. If such exposure occurred, it may have caused mild inflammation of the lower airways of the healthy horses. In experimental settings, neutrophil percentage in BALF may mildly but significantly increase in healthy horses exposed to endotoxins and hay/straw challenge (Pirie *et al.* 2002). Such an increase in neutrophil percentage may be transient for only few hours (Pirie *et al.* 2002), however the activity of recruited cells is unknown. The horses in this study were not stabled on hay/straw bedding and a window was always opened to encourage ventilation of the stable, however there are no objective data to determine exposure to aeroallergens/antigens or endotoxins. Assessing the respiratory hygiene of the stable environment and in the breathing zone of the horses (eg. exposure to dust or endotoxin) was desirable and would have been beneficial for the current study, however equipment required for these investigations were not available for this study. In addition, ammonia (NH₃) is commonly present in the environment of stables (Dixon *et al.* 1995b) and assessment of the

concentration of NH_3 in the stable would have been useful to contribute to the understanding of EBC pH as it is possible that NH_3 influences the acid-base balance of the lower airways in horses as found previously in humans (Wells *et al.* 2005). The effect of respirable environmental factors on the lower airways of the horses in the control group is unknown as no BALF cytology was performed in these animals.

Despite the limitations of the current study, the findings suggest that analysis of EBC H_2O_2 concentration and pH are not useful for detection of mild-moderate neutrophilic LAI. Further studies are required to determine whether quantification of H_2O_2 concentration and pH in EBC are useful for detection of more marked LAI.

CHAPTER 5 – CONCENTRATION OF H₂O₂ AND pH IN EBC OF HEALTHY HORSES AND HORSES WITH LAI

5.1 Introduction

Exhaled breath condensate (EBC) has been studied widely in human patients with inflammatory diseases of the lower airways, including asthma. Numerous compounds are present in EBC from humans including hydrogen peroxide (H₂O₂), nitric oxides, adenosine, arachidonic acid metabolites, cytokines, leukotrienes, 8-isoprostane and ammonia (Horvath *et al.* 2005). Exhaled breath condensate H₂O₂ concentration is considered a suitable parameter for frequent monitoring of patients with respiratory disease such as asthma and COPD (Loukides *et al.* 1998; Loukides *et al.* 2002; Horvath *et al.* 2005), despite the reported coefficient of variation for the parameter (van Beurden *et al.* 2002a).

The concentration of H₂O₂ in EBC of horses with RAO has been reported; a significant relationship between the concentration of H₂O₂ in EBC and both the severity of clinical signs and BAL neutrophil count and ratio was found (Deaton *et al.* 2004b). In that study, the concentration of H₂O₂ in EBC was higher in affected horses during exacerbation of RAO than during a period of remission and also in comparison to healthy controls (Deaton *et al.* 2004b). However, in a subsequent study performed by the same authors, no relationship between concentration of H₂O₂ in EBC and presence of oxidative stress in the lower airways of RAO horses after environmental challenge was found and no significant increase in EBC H₂O₂ concentration in the horse was recorded (Deaton *et al.* 2005a). Similarly Wyse *et al.* (2005) found no significant difference in EBC H₂O₂ concentration before and after environmental challenge in horses with RAO. The data presented in Chapter 4 of this thesis are suggestive of high variability in EBC H₂O₂ concentration in both healthy controls and horses

with mild LAI. Further in the study of Chapter 4, no significant difference was found in EBC H₂O₂ concentration between the control and LAI groups. Hydrogen peroxide is produced primarily by activated inflammatory cells in the airways as a consequence of oxidative stress (Babior 2000). In humans H₂O₂ is also produced by epithelial cells in the conducting airways and expiratory flow rates have been shown to influence its concentration in EBC (Schleiss *et al.* 2000). Potential limitations to the use of H₂O₂ for detection of LAI are that it is photo- and thermo-sensitive, extremely reactive towards a wide selection of materials and subject to rapid degradation. Further, a wide inter-day coefficient of variation for EBC H₂O₂ in healthy humans has been reported (van Beurden *et al.* 2002a) and may be due to chemical reactivity and instability or circadian rhythms of H₂O₂ secretion (Nowak *et al.* 2001). In contrast, other authors have described good correlation between EBC H₂O₂ concentration and the presence and severity of asthma in humans and response to treatment (Antczak *et al.* 2000; Loukides *et al.* 2002).

Similarly, EBC pH has been demonstrated to be a robust and reliable indicator of lower airway inflammation in humans affected by asthma or COPD (Kostikas *et al.* 2002; Vaughan *et al.* 2003; Borrill *et al.* 2005). In addition, the degree of acidification in the lower airways has been shown to be correlated to the presence and severity of asthma in affected human patients (Hunt *et al.* 2000). Acidification of the lower airway lining fluid detected in EBC appears to be implicated in the pathophysiology of asthma in humans, as a low pH may predispose to asthma and reflects an abnormality in the regulation of non-volatile constituents in the lower airways (Hunt *et al.* 2000). The pH of EBC in healthy humans has been found to be very stable overtime with reported mean intraweek and intraday coefficients of variation of 4.5% and 3.5% (Vaughan *et al.* 2003). It is unknown whether there is an association between pH of EBC and the health/disease status of the lower airways of horses.

The aims of this study were:

1. to determine whether significant differences in EBC H_2O_2 concentration and pH are present between healthy horses and horses with LAI (as defined by increased neutrophil percentage in BALF)
2. to investigate if a relationship between H_2O_2 concentration and pH in EBC exists in both healthy horses and horses with LAI. It is possible that an increase in H_2O_2 concentration may indicate oxidative stress and be related to acidification of the BALF as suggested in studies of human asthma (Hunt *et al.* 2000).

5.2 Materials and Methods

5.2.1 Animals

Thirty-one horses were used in the study. Horses were divided into three groups depending on housing and respiratory tract health as determined by the presence or absence of a history consistent with LAI, with/without BALF cytology results.

Group 1: a stable control group comprised of 19 horses (5 mares, 14 geldings; 9 Cobs, 4 Warmblood, 3 Ponies, 2 Thoroughbred crosses, 1 Anglo-arab cross; age: 11 ± 5 years) that did not have a history of respiratory disease. All horses underwent thorough clinical examination, including auscultation of the lungs during forced rebreathing. No abnormal bronchoalveolar or tracheal sounds were detected in any horse (apart from #9 and #13 which were subsequently excluded from the study). Broncho-alveolar lavage was not performed for ethics and welfare reasons. Fourteen horses were from the same yard and were stabled on wood shavings in a traditional stall, where the ceiling communicated between stalls and the upper section of the stable door was opened at all

times to maximise ventilation. The remaining five horses of the group were stabled in modern stables with good ventilation on paper bedding and were fed soaked hay. None of the horses had received any medication during the previous 4 months. In addition, the horses were not administered any medications for at least 72 hours prior to the beginning of the study, as declared by the owners. Signalment and management data for the horses in this group are provided in Table 5.1.

Table 5.1: Signalment, stable bedding used and feeding of control horses housed in stables

	Age (years)	Gender	Breed	Bedding	Diet
Horse 1	15	M	TB X	Paper	Soaked hay
Horse 2	5	G	TB X	Paper	Soaked hay
Horse 3	4	G	WB X	Paper	Soaked hay
Horse 4	16	G	Pony	Wood-shavings	Dry hay
Horse 5	7	G	Cob X	Wood-shavings	Dry hay
Horse 6	6	G	Cob X	Wood-shavings	Dry hay
Horse 7	12	G	Irish Cob	Wood-shavings	Dry hay
Horse 8	15	G	Welsh Cob X	Wood-shavings	Dry hay
Horse 9	11	G	Anglo-Arab X	Wood-shavings	Dry hay
Horse 10	11	M	Cob X	Wood-shavings	Dry hay
Horse 11	6	G	Belgian WB	Wood-shavings	Dry hay
Horse 12	12	G	Pony	Wood-shavings	Dry hay
Horse 13	20	G	Welsh Cob X	Wood-shavings	Dry hay
Horse 14	19	M	Welsh Cob X	Wood-shavings	Dry hay
Horse 15	7	M	WB X	Wood-shavings	Dry hay
Horse 16	5	G	Welsh Cob X	Wood-shavings	Dry hay
Horse 17	20	G	Cob X	Wood-shavings	Dry hay
Horse 18	8	G	Pony	Paper	Soaked hay
Horse 19	9	M	WB X	Paper	Soaked hay

G=gelding; M=mare; TB= Thoroughbred; WB=Warmblood; X=cross

Group 2: A pasture control group included four healthy horses (2 mares and 2 geldings; 2 Warmblood and 2 New-forest ponies; age 5 ± 3 years) that lived on pasture at least 12 hours a day (Table 5.2). These horses did not have a history consistent with LAI and no evidence of LAI was

detected during physical examination, including auscultation of the lungs during forced rebreathing. Broncho-alveolar lavage was not performed from the horses of this group for ethics and welfare reasons. For all horses, EBC was collected immediately after return from pasture. None of the horses had received any medication during the previous 4 months. In addition, the horses were not administered any therapeutic preparation for at least 72 hours prior to the beginning of the study, as declared by the owners.

Table 5.2: Signalment and housing of pastured control horses

	Age	Gender	Breed	Pasture
Horse 20	10	Gelding	Warmblood	12h/day
Horse 21	4	Mare	Warmblood	12h/day
Horse 22	2	Gelding	New-Forest pony	24h/day
Horse 23	4	Mare	New-Forest pony	24h/day

Group 3: this group comprised of horses with LAI (LAI group) and included 8 horses (6 mares and 2 geldings; 3 ponies, 2 Thoroughbred crosses, 3 Warmblood crosses; age: 14 ± 6 years). Lower airway inflammation was confirmed by history, clinical signs (cough, exercise intolerance and nasal discharge) and BALF cytology (neutrophils > 5%). Horses were stabled on paper bedding and fed soaked hay, in stalls with good ventilation.

Table 5.3: Signalment, history, clinical signs and BALF cytology of horses with lower airway inflammation (LAI group)

Signalment		History and clinical signs	BAL cytology	
Horse 24	TB X Mare 15 yo	Nasal discharge occasional cough	Neutrophils	18%
			Macrophages	23%
			Lymphocytes	54%
			Mast cells	5%
Horse 25	Pony Mare 22 yo	Occasional cough prolonged recovery time from exercise	Neutrophils	13%
			Macrophages	41%
			Lymphocytes	35%
			Mast cells	11%
Horse 26	TB X Mare 24 yo	Occasional cough nasal discharge	Neutrophils	11%
			Macrophages	33%
			Lymphocytes	45%
			Mast cells	1%
Horse 27	WB X Mare 13 yo	Exercise intolerance prolonged recovery time from exercise occasional cough	Neutrophils	15%
			Macrophages	30%
			Lymphocytes	50%
			Mast cells	5%
Horse 28	WB X Geld 11 yo	Cough RAO exacerbation (12h earlier) received IV Dexamethasone increased expiratory effort	Neutrophils	29%
			Macrophages	36%
			Lymphocytes	26%
			Mast cells	6%
Horse 29	NF Pony Geld 14 yo	RAO previously diagnosed ended inhalatory steroid treatment (2 weeks earlier) in remission	Neutrophils	22%
			Macrophages	6%
			Lymphocytes	62%
			Mast cells	10%
Horse 30	NF Pony Mare 9 yo	RAO previously diagnosed ended inhalatory steroid treatment (2 weeks earlier) in remission	Neutrophils	8%
			Macrophages	24%
			Lymphocytes	60%
			Mast cells	8%
Horse 31	WB X Mare 6 yo	Increased respiratory effort tachypnea cough nasal discharge	Neutrophils	64%
			Macrophages	19%
			Lymphocytes	14%
			Mast cells	3%

RAO= recurrent airway obstruction; TB=Throughbred; WB=Warmblood; NF= New Forest; y.o.=years old

5.2.2 Collection of EBC Samples and Analysis

Stable and Pasture control groups: One EBC was collected from each horse using the methods described in paragraph 2.1. Each sample was collected over a period of 10 minutes, stored immediately on ice after collection and thawed immediately prior to analysis. Concentration of H₂O₂ and pH in each sample were determined within one hour of collection.

LAI group: presence of LAI was confirmed by BAL cytological examination. Exhaled breath condensate was collected immediately prior to the BAL procedure and the collected sample was stored immediately on ice. Concentration of H₂O₂ and pH in each sample were determined within one hour of collection.

5.2.3 Statistical analysis

One-way ANOVA was used to detect significant differences ($p < 0.05$) in EBC H₂O₂ concentration, EBC pH, respiratory rate and EBC volume collected between the three groups. Pearson's correlation coefficients were calculated to determine whether correlations between EBC H₂O₂, EBC pH, horse age, EBC volume produced and horse respiratory rate were present. Relationships between BAL neutrophil ratio and EBC H₂O₂ concentration and pH were studied in the LAI group using the Pearson's correlation rank. For the calculation of the Pearson's correlation coefficients stable control group and pasture control group are combined in a unique group.

5.3 Results

A total of 31 samples of EBC were collected (1 sample from each horse in the study). A tracheal rattle was heard in two horses in the stable control group during forced rebreathing and the EBC samples from these two

horses were excluded from the study (Table 5.6). Sample collection was tolerated by all horses in the study except horse 10 where the procedure was suspended after 5 minutes (0.6ml collected) because the horse became excited and developed a markedly raises respiratory rate (>30breaths/min) and an irregular respiratory rhythm. No significant difference was present between groups for respiratory rates during collection ($p=0.734$) or volume ($p=0.841$) of EBC collected (Table 5.4).

Table 5.4: Mean and standard deviation (SD) of respiratory rate and volume of EBC collected from the Stable control group, Pasture control group and lower airway inflammation (LAI) group.

	Respiratory Rate (brpm)		Volume collected (ml)	
	Mean	SD	Mean	SD
Stable control group	14	5	2.0	1.3
Pasture control group	13	3	1.8	0.2
LAI group	15	4	1.6	1.1

The mean (\pm SD) EBC H_2O_2 concentration was $4.0\pm 2.9\mu M$ (median: $3.3\mu M$, range: $0.9-12.9\mu M$) in the stable control group, $3.3\pm 0.5\mu M$ (median: $3.3\mu M$, range: $2.6-5.4\mu M$) in the pasture control group and $3.9\pm 3.0\mu M$ (median: $2.94\mu M$, range: $9.7-0.9\mu M$) in the LAI group. The mean \pm standard deviation EBC pH was 5.77 ± 0.55 (median: 5.77, range: 6.70-4.78) in the stable control group, 5.72 ± 0.21 (median: 5.80, range: 5.41-5.88) in the pasture control group and 5.31 ± 0.62 (median: 5.38, range: 4.41-6.10) in the LAI group (Table 5.5).

Table 5.5: Mean and SD of H_2O_2 concentration and pH of EBC collected from the Stable control group, Pasture control group and lower airway inflammation (LAI) group

	H_2O_2 (μM)		pH	
	Mean	SD	Mean	SD
Stable control group	4.0	2.9	5.77	0.55
Pasture control group	3.3	0.5	5.72	0.21
LAI group	3.9	3.0	5.31	0.62

No significant difference was found between groups for EBC H₂O₂ concentration (p=0.895) (Figure 5.1) or EBC pH (p=0.188) (Figure 5.2). Summary data of EBC H₂O₂ concentration and pH for each group are provided in Table 5.5. Data of EBC H₂O₂ concentration and pH for each horse is provided in Table 5.6.

Table 5.6: EBC H₂O₂ concentration and pH, respiratory rate (RR; breaths per minute) and volume of EBC collected from the Stable control group, Pasture control group and horses in the group with lower airway inflammation (LAI) (*: horses 9 and 13 were excluded for detection of abnormal tracheal sound during auscultation)

GROUP	HORSE	H ₂ O ₂ (μM)	pH	RR	Volume (ml)
Stable Control Group	Horse 1	7.7	4.95	8	2.5
	Horse 2	4.4	5.49	15	2.0
	Horse 3	4.8	4.78	13	0.8
	Horse 4	2.9	5.81	12	1.0
	Horse 5	12.9	5.55	10	0.8
	Horse 6	3.4	5.70	10	1.0
	Horse 7	1.5	5.75	8	1.5
	Horse 8	2.3	5.77	10	1.5
	Horse 9*	10.8	5.48	14	0.8
	Horse 10	4.1	6.40	24	0.6
	Horse 11	0.9	5.86	10	0.8
	Horse 12	1.4	6.59	24	1.0
	Horse 13*	6.4	6.40	16	1.8
	Horse 14	3.1	5.83	12	1.5
	Horse 15	3.3	6.70	22	3.0
	Horse 16	5.4	6.33	16	3.0
	Horse 17	6.0	6.12	14	3.5
	Horse 18	1.4	5.15	16	2.8
	Horse 19	3.2	5.24	10	4.8
Pasture control group	Horse 20	3.4	5.82	8	1.7
	Horse 21	3.5	5.41	12	1.7
	Horse 22	3.8	5.88	14	1.7
	Horse 23	2.6	5.78	16	2.1
LAI group	Horse 24	0.9	5.53	8	1.5
	Horse 25	2.4	5.64	12	1.0
	Horse 26	9.7	5.83	20	4.0
	Horse 27	3.3	6.10	12	1.8
	Horse 28	6.3	4.85	18	1.0
	Horse 29	2.4	4.84	18	0.5
	Horse 30	2.6	4.41	16	1.4
	Horse 31	4.8	5.22	20	3.0

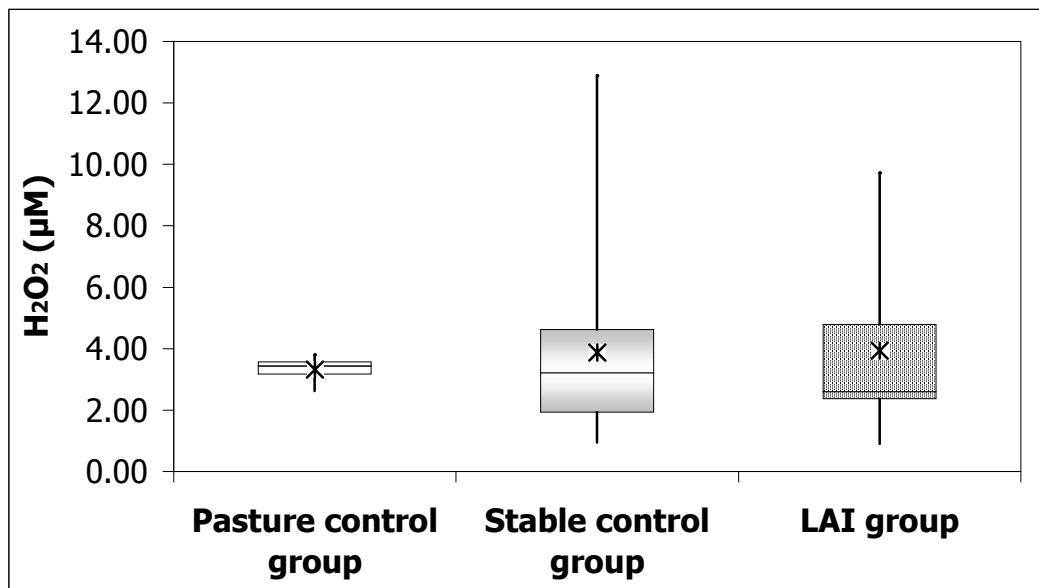


Figure 5.1: Boxplot of EBC H_2O_2 concentration for each group; no statistically significant difference between groups was present ($p=0.895$); * = mean

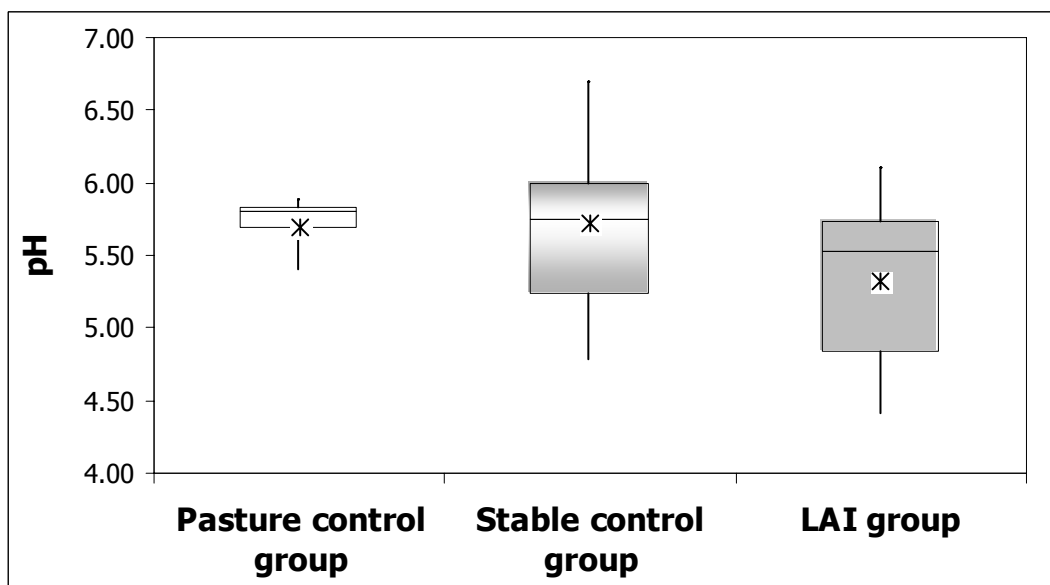


Figure 5.2: Boxplot of EBC pH of the pasture control group, stable control group and lower airway inflammation group; no significant difference between the three groups was detected ($p=0.188$); * = mean

Figure 5.3 and Figure 5.4 demonstrate the distribution of EBC pH and H_2O_2 concentration in the control and LAI groups respectively. No significant correlation was found between concentration of H_2O_2 and pH in EBC obtained from healthy horses at pasture or in stables (Figure 5.3) and horses with LAI (Figure 5.4).

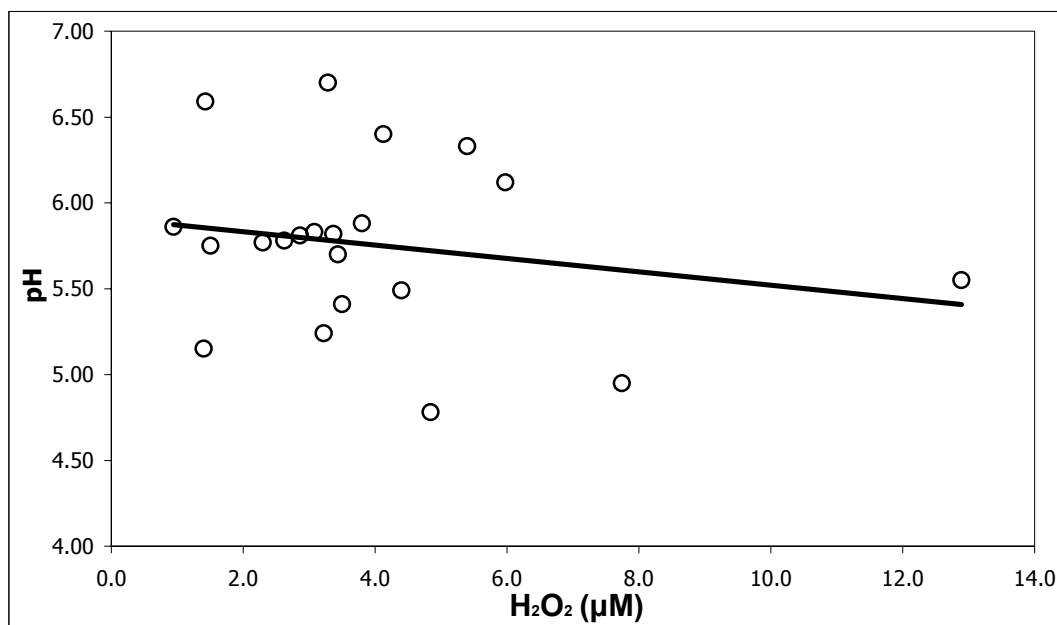


Figure 5.3: Distribution of H₂O₂ concentration and pH in EBC of healthy horses; there was no correlation between H₂O₂ concentration and pH (Pearson's correlation coefficient=-0.205; p=0.372)

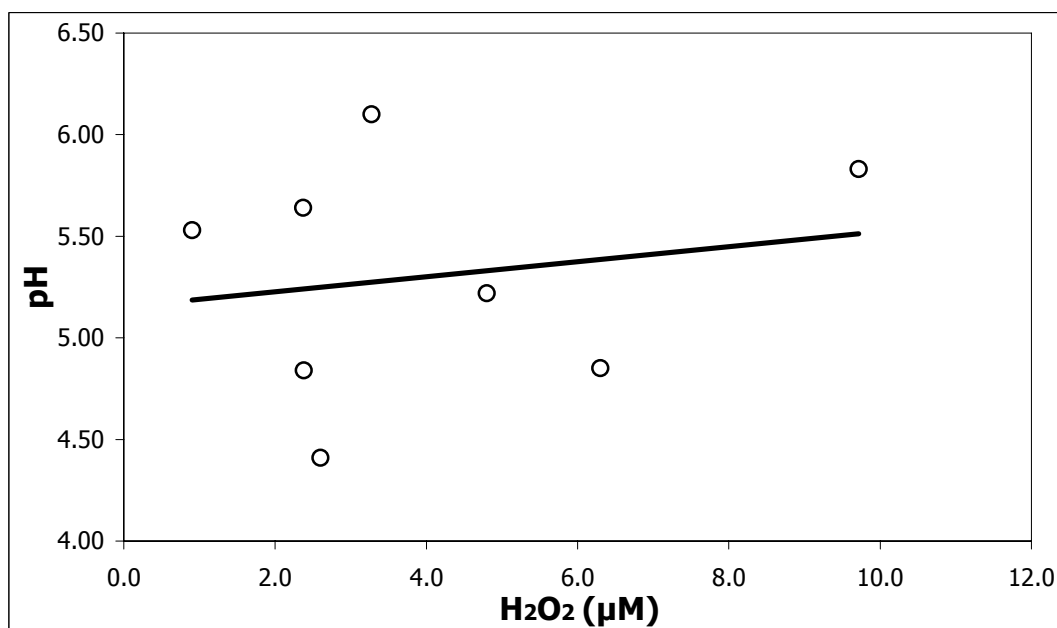


Figure 5.4: Distribution of H₂O₂ concentration and pH and trend line in horses with lower airway inflammation; no correlation was detected (Pearson's correlation coefficient=0.183; p=0.665)

Data regarding the results of Pearson's correlation analyses for healthy horses and horses with LAI are provided in Tables 5.7 and 5.8 respectively. The only significant correlation was between EBC pH and

respiratory rate in healthy horses (Pearson's correlation coefficient=0.631; $p=0.002$) (Figure 4.5).

Table 5.7: Pearson's correlation coefficient for variables in healthy horses; *:statistically significant

	Pasture control group, stable control group			
	Resp Rate	Volume	H ₂ O ₂	pH
Age	-0.113 0.667	0.083 0.055	-0.006 $p=0.980$	0.145 $p=0.530$
Resp Rate		0.033 $p=0.868$	-0.001 $p=0.996$	0.631 $p=0.002^*$
Volume			-0.182 $p=0.430$	-0.063 $p=0.785$
H ₂ O ₂				-0.205 $p=0.372$

Table 5.8: Pearson's correlation coefficient for variables in horses with lower airway inflammation

	LAI group			
	Resp Rate	Volume	H ₂ O ₂	pH
Age	-0.176 0.920	0.204 0.770	0.419 $p=0.349$	0.635 $p=0.125$
Resp Rate		0.033 $p=0.868$	0.752 $p=0.051$	0.502 $p=0.251$
Volume			0.739 $p=0.057$	-0.375 $p=0.487$
H ₂ O ₂				0.191 $p=0.682$

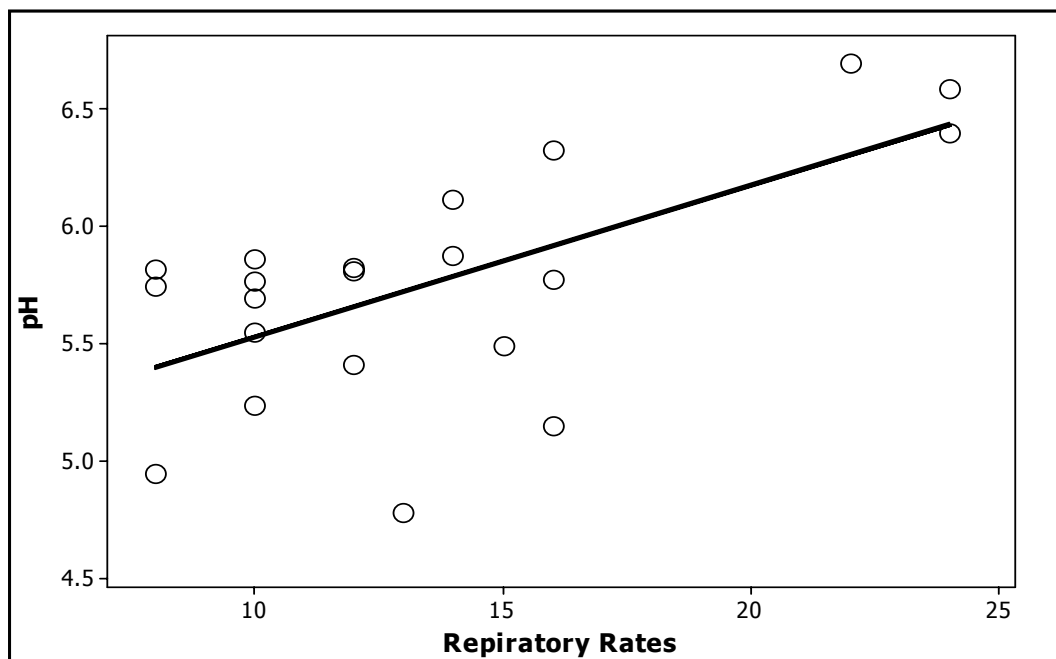


Figure 5.5: Relationship between respiratory rate and EBC pH in healthy horses; a significant relationship was detected using a Pearson's correlation coefficient (0.631; $p=0.002$).

5.4 Discussion

No significant differences in either EBC H_2O_2 concentration or pH were found between the three groups of horses in the current study. In contrast, Deaton *et al.* (2004) found that horses with RAO had a significantly higher concentration of H_2O_2 in EBC than healthy control horses. In the study by Deaton *et al.* (2004) horses with severe clinical signs of RAO were used. In the current study horses with LAI had mild clinical signs and may explain the lack of a difference in EBC H_2O_2 concentration in EBC between healthy horses and horses with LAI. In humans, H_2O_2 concentration in EBC appears to be a useful marker of airway inflammation. A significant correlation between EBC H_2O_2 concentration and severity of asthma has been documented (Loukides *et al.* 2002; Kharitonov and Barnes 2004). Asthmatic children have a lower concentration of H_2O_2 in EBC during remission or following treatment with corticosteroids, compared to the acute phase of the disease (Kharitonov and Barnes 2004).

It would have been preferable to have performed BAL in the control horses of the current study to allow more objective differentiation between control and LAI groups by the use of respiratory cytology rather than sole reliance on history and physical examination. It is possible that some horses in the stable or pasture control groups had subclinical LAI that was not detected by history and physical examination alone. However, for ethics and welfare reasons, BAL was not performed in healthy horses. The absence of respiratory disease in the history of each horse and absence of clinical signs of LAI were considered acceptable to identify horses likely free of lower airway disease. It has been shown that examination of historical information can identify horses with RAO (Hotchkiss *et al.* 2006; Ramseyer *et al.* 2007), however this approach is likely to be less sensitive to identify those horses with IAD. In addition, it is possible that one or more of the control horses that had high H₂O₂ concentration or low pH in EBC could have had an upper airway disease (e.g. sinusitis, pharyngitis, laryngitis) that altered these parameters. Human patients with sinusitis have been found to have increased EBC H₂O₂ concentration for up to two weeks following resolution of clinical signs (Jobsis *et al.* 2001). The effect of upper airway pathology on EBC H₂O₂ and pH, the site of production of H₂O₂ detected in EBC and the proportion of H₂O₂ produced from the upper and lower respiratory tract in the horse are all unknown. While no evidence of upper respiratory tract disease was detected on physical examination, upper airway endoscopy and radiography were not performed in control horses. Moreover, the horses in the LAI group showed only mild clinical signs of LAI, namely nasal discharge, occasional cough and/or prolonged recovery time from exercise. No increased respiratory effort at rest was present and a mild seromucous nasal discharge was the only clinical abnormality in some horses. Two horses with a history of RAO had no clinical signs at the time of collection of EBC and BALF (BAL was performed as a component of

clinical reassessment after prior diagnosis of the disease). It is possible that these factors, individually or collectively, reduced the differences in H₂O₂ concentration and pH of EBC between control horses and horses with LAI. Further, it is possible that including horses with more severe clinical signs may have resulted in significant differences in EBC H₂O₂ and pH between groups as reported by Deaton et al (2004a).

Collection of EBC was tolerated well by all but one of the horses. One mare did not tolerate prolonged breathing through the mask possibly as a result of the noise generated by the movement of the one-way valve of the mask adjacent to the nose of the horse. After 5 minutes the procedure was discontinued as the excitation of the horse caused a marked increase in the respiratory rate (>30) with an irregular rhythm. Some horses in the study required a few minutes to get accustomed to the mask and EBC collection was commenced when the respiratory rates returned to the rate prior to application of the mask. In most situations one operator was sufficient to undertake EBC collection from the horses as the sampling procedure was simple and easy to perform. As reported in human studies of EBC collection (Griese *et al.* 2001; Mutlu *et al.* 2001) ease of collection is an advantage of this methodology that justifies studies of EBC analysis in relation to respiratory disease in horses.

An increase in the respiratory rate was considered the main indicator of horse distress during EBC collection in the current study. In human patients flow-dependency of H₂O₂ production has been determined, suggesting that H₂O₂ is produced also by the epithelial cells of the conducting airways (Schleiss *et al.* 2000). Although there is limited knowledge concerning the production of H₂O₂ in the equine airways, collection of EBC at tidal breathing was attempted in the current study. Further studies are required to assess whether expiratory flow rates can influence H₂O₂ production in equine species.

The EBC pH in the horses of the current study (mean \pm SD: 5.76 \pm 0.5, median: 5.78, range: 4.78-6.70 in healthy horses) was, on average, lower than ranges reported for plain samples from human patients with asthma (median: 6.46, range: 5.86-6.72) and healthy humans (usually greater than 7) (Leung *et al.* 2006). In the current study there was no statistically significant difference in EBC pH between stabled control, pasture control and LAI groups by the use of ANOVA ($p=0.188$). It is possible that the horses comprising the 3 groups did not have sufficient differences in airway health to induce appreciable changes in EBC pH. Whether including horses with more severe clinical signs of LAI and using control horses with confirmed normal respiratory cytology would increase the likelihood of significant differences in EBC pH, remains to be established. Studies in human medicine reported a significantly lower pH in patients affected by disease such as asthma (Hunt *et al.* 2000), COPD, bronchiectasis (Kostikas *et al.* 2002), cystic fibrosis (Tate *et al.* 2002), and acute lung injury (Gessner *et al.* 2003) compared to healthy controls. The effect inhalation of ammonia present in the air of the horses environment has on EBC pH is unknown and requires clarification. Contamination of EBC with oral ammonia is of concern to human investigators and the recommendation is to incorporate a saliva trap in the collection device to minimize contamination of EBC samples with oral ammonia and if a saliva trap is not used, the ammonia concentration in EBC should be evaluated (Effros *et al.* 2002; Horvath *et al.* 2005). However, determining the concentration of ammonia in inspired/expired air was beyond the scope of the current study.

A significant correlation between EBC pH and respiratory rate in the control horses was determined by linear regression analysis ($p=0.002$). In three control horses the respiratory rate increased slightly (just over 20 breaths/minute) due likely to mild excitement. In these horses the pH of

EBC was higher than in horses with a lower respiratory rate and this likely contributed to the observed correlation. In humans, no relationship between respiratory pattern and EBC pH has been found, suggesting that flow dependency for EBC pH does not occur (Vaughan *et al.* 2003). In the current study, EBC pH and respiratory rates in horses with LAI were not correlated. Hydrogen peroxide concentration in EBC was not significantly correlated to the respiratory rate during EBC collection ($p=0.051$) and volume of EBC collected ($p=0.057$) in the LAI group, however trends were present. The trend between EBC H_2O_2 concentration and respiratory rate may suggest that EBC H_2O_2 concentration may be flow dependent, however numbers of horses in the study were limited. Utilisation of greater numbers of horses and inclusion of greater diversity of respiratory rates (e.g. before and after moderate exercise) may provide useful data to establish if H_2O_2 concentration and pH in EBC are flow dependent. Recently, moderate intensity exercise was shown to cause a significant increase in EBC pH in healthy humans (Riediker and Danuser 2007) and it was speculated that exercise can induce oxidative stress on the lower airways. The effect of exercise on EBC pH in healthy horses and horses with LAI is unknown. However, exercise in horses, particularly when breathing cold, dry air can lead to lower airway inflammation (Davis *et al.* 2007). It remains possible the effect of exercise in horses could be assessed by measuring EBC H_2O_2 concentration and pH, if the effect of exercise are sufficient to trigger an inflammatory response in the lower airways. However, it would remain difficult to assess whether inflammation is a consequence of exercise or if there is a flow dependency of H_2O_2 and pH when using exercise.

CHAPTER 6 – GENERAL DISCUSSION AND CONCLUSIONS

6.1 Collection and Analysis of EBC in Horses

The results of the current studies indicate that the equipment and methodology used to collect EBC from standing non-sedated horses were reliable and repeatable. The equipment used is portable and suitable for stable-side collection and was adapted from that described by Wyse *et al.* (2005). The methodology for collection of EBC is simple and a single operator was sufficient to undertake EBC collection from the horses. All but one horse involved in the study tolerated the collection procedure. Overall, horses appeared to become more accustomed to the collection procedure with successive collections. Respiratory rate and rhythm of horses were minimally or not affected by the procedure. In addition to the minimal personnel requirements for collection of EBC, in the current study there was no requirement for physical or chemical restraint of any horse. A total of 121 samples from 33 horses were collected throughout the course of the study, suggesting the method used was safe, rapid and simple. Documentation of respiratory rate of each horse and collection of the EBC sample during basal respiration were considered important because H_2O_2 concentration in EBC is potentially influenced by respiratory flow rates as suggested by human studies (Schleiss *et al.* 2000). Flow-dependency of H_2O_2 production in EBC of human patients has been documented, suggesting that a portion of H_2O_2 is produced by the epithelial cells of the conducting airways during periods of increased air-flow (Schleiss *et al.* 2000). The internal diameter of the tubing used to collect EBC was approximately 5cm and two valves were used to minimize resistance to airflow in the system. Increased breathing resistance could have potentially caused distress in some animals and affected respiratory flow rates. On account of the unchanged behaviour and unaffected

respiratory rates of the horses during the sampling procedure it is concluded that this technique is very well tolerated and is suitable for EBC stall side collection. The materials used to build the collection system were chosen for their inert properties. The condensation chamber was constructed of polypropylene, in compliance to the official recommendation for EBC collection (Horvath *et al.* 2005). The disinfection protocol used throughout the study was evaluated and found not to affect the concentration of H₂O₂ or the pH.

The gold standard for the assessment of LAI in horses is cytological examination of BALF (Christley and Rush 2007; Lavoie 2007). Bronchoalveolar lavage is an invasive procedure, requires sedation and physical restraint of the horse and can cause irritation of the respiratory tract. Horses may cough violently and demonstrate signs of discomfort during the procedure. Although the irritation (and occasionally distress) of this procedure can be alleviated by sedation and instillation of local anaesthetic in the lumen of the trachea, many horses still cough during the procedure. The trauma induced by the passage of the BAL tube/endoscope and lavage with saline may induce inflammation of the lower airways. The procedure can stimulate the migration of inflammatory cells into the airways and result in an increase in neutrophil percentage in repeated BAL samples obtained from the same lung segment at least 48 hours later (Sweeney *et al.* 1994). Sweeney *et al.* (1994) reported that neutrophil percentage in BALF from healthy horses increased from a mean of 2.3% at the initial sample to a mean of 27.3% in a second sample obtained 48 hours later. This finding suggests that BAL is not suitable for short term monitoring of lower airway health in horses both from a welfare perspective and because of difficulties in interpreting cytological results. A further limitation of cytologic examination of BALF is that while evidence of the number of inflammatory cells (principally neutrophils) in the lower airways is provided, it does not give any information regarding

the activation status of inflammatory cells. A potential advantage of EBC analysis is that evidence of the functional activity of granulocytes may be obtained. Furthermore, due to the non-invasive nature of the procedure, collecting multiple EBC samples over a short period of time will not result in any airway inflammation; hence EBC analysis is suitable for frequent sampling and may prove ideal for monitoring horses with respiratory disease (Wyse *et al.* 2005).

The hazard related to the use of liquid nitrogen is a possible limitation of the practicality of EBC collection due to specific requirements for handling of liquid nitrogen. In the current study, liquid nitrogen was chosen to obtain the lowest possible temperature in the condensation chamber. Liquid nitrogen needs to be handled with considerable caution to avoid contact with the skin of personnel and horses. The fumes of liquid nitrogen can be harmful and the liquid must be handled in an environment with good air circulation. Furthermore, liquid nitrogen is not necessarily accessible and there are considerable security issues related to its transport. However, many veterinary surgeons are equipped for its transportation since it is widely used, e.g. to preserve frozen semen and for cryotherapy. In the current study liquid nitrogen was mixed with ethanol because ethanol remains as a liquid at very low temperatures (melting point -114.3°C), is inexpensive and is readily available. The rationale to mix liquid nitrogen and ethanol is that the obtained slush did not produce any fumes and maintained the required temperature range for several hours (after 3 hours at room temperature, the temperature of the slush in the polystyrene container was still less than -80°C). In addition, these low temperatures were chosen to maximize the volume of EBC collected over a period of ten minutes. Further, this collection temperature is in compliance with the official recommendation to freeze samples at $\leq -70^{\circ}\text{C}$ immediately after collection until analysed (Horvath *et al.* 2005), as lower temperatures achieve a better preservation of H_2O_2 .

(van Beurden *et al.* 2002b). In preliminary studies, temperatures achieved using ice to surround the condensation chamber were found to be insufficient for EBC collection (less than 0.4ml of EBC were obtained), while the liquid nitrogen/ethanol mixture allowed collection of an adequate volume of EBC for laboratory analysis of both H₂O₂ and pH.

The concentration of H₂O₂ detected in the EBC samples of the current study tended to be higher than those reported in other studies performed in horses (Deaton *et al.* 2004a; Wyse *et al.* 2005; Deaton 2006). In the studies by Deaton *et al.* (2004a) the temperature of the collection chamber was ~0°C and the use of different temperatures for the collection has been found to affect EBC H₂O₂ concentration (Goldoni *et al.* 2005). The difference between this study and the study of Deaton *et al.* (2004a) could be due to different collection temperatures. It is possible that using a low temperature ($\leq -80^{\circ}\text{C}$) improves the preservation of H₂O₂ and may be preferable for collection of EBC. However, the effect of collection temperature and EBC H₂O₂ concentration has been studied in human patients and a decreased H₂O₂ concentration in samples collected at lower temperatures (at -10°C compared to +5°C) was reported, possibly due to dilutional effects of a larger volume of EBC collected at the lower temperature (Goldoni *et al.* 2005). However, in humans the absolute amount of H₂O₂, obtained by multiplying its concentration by the total volume of EBC, was higher collecting at lower temperatures (Goldoni *et al.* 2005). The effect of collection temperature on measured H₂O₂ concentration in EBC samples is unknown and was not investigated in the current study. It is unlikely the difference in H₂O₂ concentration between the current study and that of Deaton *et al.* (2004a) was due to methodological differences in the protocol for the quantification of H₂O₂, as the assay used between these studies was the same.

The results of the studies discussed in Chapter 2 indicate that the measurement of the pH of EBC can be problematic as a period of up to 20 minutes may be required to obtain a stable reading. In human medicine the instability of the EBC pH is well recognised and is attributed to volatile substances present in EBC (Hunt *et al.* 2000). Carbon dioxide is believed to be the main cause of instability, but there is no official recommendation in human medicine as to whether pH of EBC should be measured with or without deaeration (Horvath *et al.* 2005). Some authors have attempted to standardise for CO₂ partial pressure and reported a better stability than that achieved by deaeration with argon (Kullmann *et al.* 2007). In the current study various methods of stabilising the reading of EBC pH were studied. Those obtained from the plain samples were the most repeatable and reliable in comparison to human studies where deaeration with argon or standardisation with CO₂ give the most repeatable measurements (Borrill *et al.* 2006; Kullmann *et al.* 2007). This method was selected for subsequent investigation in the current study. Determination of all the components present in equine EBC would be useful as this would allow a better understanding of the mechanisms leading to instability of pH and whether any interactions between components are likely to occur. However, such investigation were beyond the scope of the current study.

A polypropylene (PP) condensation chamber was a component of the equipment used in the current study. Polypropylene is an established material for EBC collection in human medicine (Horvath *et al.* 2005). This material is a hypo-reactive plastic that is very stable at high and very low temperatures. In addition, PP is ideal for stall-side collection, as it is light, impermeable and resistant to breakage. In contrast, a glass or pyrex chamber would be susceptible to breaking and if breakage occurred this could compromise the safety of the operator or of the horse.

The method of collection used in the current studies could also be useful to investigate other markers of inflammation (e.g. cytokines, leukotrienes, ammonia, nitric oxides, proteins, etc.) that have not been investigated in equine EBC to date. In human studies expression of many markers of inflammation (IL-4, IL-6, IL-8, γ -INF, 8-isoprostane) and other substances (ammonia, CO₂, carbonic acid, lactate) in EBC has been correlated with the presence of asthma, COPD and acute lung injury (Shahid *et al.* 2002; Gessner *et al.* 2003; Carpagnano *et al.* 2004; Ko *et al.* 2006).

A previous study found correlations between the degree of bronchoalveolar neutrophilia, clinical signs of RAO and EBC H₂O₂ concentration (Deaton *et al.* 2004a). In contrast, no significant difference was found in H₂O₂ concentration EBC between horses with or without LAI in the current study. For ethics and welfare reasons we did not perform a BAL and cytological analysis of BALF in control horses to confirm the absence of LAI. Further, horses with confirmed LAI in the current study had only mild clinical signs of respiratory disease. It is possible that horses with subclinical LAI were included in the healthy control group and therefore biased the results towards a non-significant difference between healthy and LAI groups. However, the results of the current study indicate that considerable variability exists in H₂O₂ concentration in EBC from individual horses both within and between days. This variability limits the usefulness of EBC H₂O₂ concentration as a diagnostic tool for the investigation of lower airway inflammation in individual horses, as it would not be possible to ascertain whether variability is the result of a physiological variation or sequela of an inflammatory process. With consideration of these findings, it is very likely that this variability minimized differences between healthy horses and those with LAI for H₂O₂ concentration. However, horses with more marked LAI could have higher EBC H₂O₂ concentration as suggested by Deaton *et al.* (2004a) and

inclusion of such horses may have differentiated horses with LAI from healthy horses more clearly.

6.2 Future Use of EBC H₂O₂ Analysis in the Horse

From the results of the current studies, EBC H₂O₂ does not appear to be useful to monitor horses with LAI, particularly when disease is mild because it would be difficult to determine if a difference in EBC H₂O₂ concentration is related to airway disease or physiological variations. Further investigations are required to determine whether concentrations of this putative marker of airway inflammation increase before the development of clinical signs of airway inflammation in horses, e.g. during exacerbations of RAO. If such a relationship exists, determination of H₂O₂ concentration in EBC would be useful to achieve an earlier diagnosis of disease, allowing prompt initiation of therapy, reduced distress for the animal and expense for the owner. In addition, to determine whether a relationship between H₂O₂ concentration and severity of airway inflammation exists it would be useful studying the effect of an appropriate and effective environmental challenge in horses with RAO. While some studies have described variation in EBC H₂O₂ concentration after environmental challenge, the magnitude of the challenge was variable between studies and unlikely to be sufficient to induce considerable increase in EBC H₂O₂ concentration and conflicting results were reported between studies (Deaton *et al.* 2005a; Wyse *et al.* 2005).

An inverse relationship has been described between EBC H₂O₂ concentration and rate of airflow in the airways in humans, supporting the theory that a large amount is produced within the conducting airways (Schleiss *et al.* 2000). Monitoring air flow during collection of EBC from horses could be a possible solution for better standardization of data collection and analysis. In human studies this has been achieved by

placing a flow meter in the collection system and making patients breathe at a determined flow rate (Schleiss *et al.* 2000). Standardising the respiratory rate would not be possible in standing horses, however monitoring the expiratory flow rate could permit greater appreciation of effect of flow rate on EBC H₂O₂ concentration and a more objective assessment of this marker of inflammation in this species. Collection of EBC from horses in the current study was performed at tidal breathing in non-sedated horses that were permitted to become accustomed to the collection procedure. It is likely that monitoring the expiratory flow rates with flow-meters would allow a better understanding of the physiology of H₂O₂ release in EBC. Most portable flow-meters available commercially are too small (1cm internal diameter) for the use in horses and are expensive and investigation of the use of flow metres was beyond the scope of this thesis.

6.3 Future for the Use of EBC pH Analysis in the Horse

In the current study, EBC pH was found to have a good repeatability for both intra-day and inter-day measurements, comparable to the magnitude of variation described in human medicine (Vaughan *et al.* 2003; Borrill *et al.* 2005). Good repeatability is a fundamental requirement for the development of a test for diagnostic purposes and the daily monitoring of patients with LAI.

The ideal method to measure EBC pH remains an unresolved topic of scientific debate in human medicine and many techniques have been described to improve pH stability of EBC samples. The principal limitation of the measurement of EBC pH in horses is the time necessary for the reading to stabilize (up to 20 minutes). However, good repeatability was observed when the same time period for measurement was provided. Preparation of EBC samples with argon deaeration or CO₂ did not induce a

quicker reading but also made measurements less repeatable. Given that pH in equine EBC samples (mean 5.65; range 5.08-6.02) is considerably lower than the pH in human EBC samples (mean 7.7, range 7.4-8.8) (Vaughan *et al.* 2003) and measurement is more repeatable without deaeration or standardization with CO₂, it is possible that differences in the composition of EBC are present between horses and humans. Environmental factors may also induce differences in EBC between horses and humans. Ammonia is known to be present in the stable environment at high concentrations (Seedorf *et al.* 2007) and it is possible that it may influence EBC pH. However, ammonia concentration in the horses' environment was not measured in this study as equipment required for collection and measurement of this gas were not available.

Future studies aimed to identify the constituents of equine EBC may contribute to an improved understanding of the physiology of EBC formation and the pathophysiology of lower airway inflammation. Furthermore, these studies could also provide information useful to optimize the measurement methods of EBC H₂O₂ concentration and pH.

In the current study there was no statistically significant difference in EBC pH between healthy horses and those with LAI. However a trend for a decrease on EBC pH in horses with LAI was present (p=0.079) and this lack of significance could have been influenced by the small numbers in each group, differing environmental conditions between groups and absence of BALF cytology data from the control animals. Further studies with categorisation of both healthy horses and those with LAI by BAL cytology and the inclusion of horses with marked clinical signs in the LAI group could help determine whether measurement of pH of EBC is a useful screening test for LAI in horses. In addition, studies assessing the response to effective antigenic challenge in horses with RAO would help to assess the usefulness of EBC pH to monitor horses with this condition and

whether this analyte could provide an earlier diagnosis for prompt therapeutic intervention before the progression of clinical signs.

Airway acidification has been shown to directly relate with disease status in human patients (Hunt *et al.* 2000; Tate *et al.* 2002; Gessner *et al.* 2003; Niimi *et al.* 2004). Airway acidosis may have antimicrobial properties through the production of reactive nitrogen and oxygen species (Hunt *et al.* 2000). However, airway acidification reduces ciliary clearance as studies *ex vivo* have shown that a pH less than 6.5 substantially reduces or eliminates ciliary beating (Luk and Dulfano 1983) and increases mucus viscosity (Holma and Hegg 1989). These alterations to the health of the lower airways may induce reduced mucus clearance *in vivo* in asthmatic patients (Hunt *et al.* 2000).

Future possible applications of quantification of markers of lower airway inflammation in exhaled breath and EBC include provision of evidence of treatment efficacy in treatment trials (management and/or pharmacological). Several human studies have investigated the effect of corticosteroids on disease status such as asthma and COPD, and found that treatment with these drugs induced a decrease in markers of inflammation in EBC (Antczak *et al.* 2000; Kharitonov *et al.* 2002; Kharitonov and Barnes 2004). It is also possible that in horses with infectious diseases, such as viral or bacterial pneumonia, collection of EBC could allow non-invasive monitoring of the efficacy of treatment and timely detection of deterioration in the condition of a patient.

6.4 Conclusions

The measurement of H₂O₂ concentration in EBC currently appears unsuitable for detection and monitoring of horses with mild LAI, due to large intra-day and inter-day variability and lack of a significant increase in

concentrations compared to healthy horses. It remains possible that quantification of EBC H_2O_2 concentration may be a useful indicator of severity of LAI, as reported previously (Deaton *et al.* 2004b), however further studies using larger numbers of horses with naturally occurring disease are required.

While EBC pH had minimal variability within and between days, it did not appear to be a useful marker of mild LAI. Examination of horses with more severe LAI, for example acute RAO, may help to elucidate whether EBC pH is altered with airway inflammation. In human medicine a relationship between asthma pathophysiology and airway acidification has been documented (Hunt *et al.* 2000) and if a similar relationship between disease severity and airway pH exists in horses, such a finding could contribute to a better understanding of the pathogenesis of RAO.

The studies described in this thesis have shown that the collection of EBC in horses is easy to perform and well tolerated. Therefore EBC analysis is a new avenue of equine respiratory medicine with great potential for future research and/or clinical application. Further studies should include investigation of the usefulness of other markers of inflammation e.g. cytokines, leukotrienes, proteins and nitric oxides for detection and quantification of LAI. Several of these parameters have been found to be useful for the detection of LAI in humans (Gessner *et al.* 2003; Carpagnano *et al.* 2004; Franklin *et al.* 2006; Ko *et al.* 2006). In addition determination of ammonia concentrations in EB would be useful to investigate the influence of this compound on EBC pH.

In conclusion, the results of the current studies provide information on the variability of the concentrations of EBC H_2O_2 and pH in EBC obtained from both healthy horses and horses with LAI. Further a lack of difference in these parameters between healthy and disease horses was determined.

The results of these studies will serve to provide information upon which further studies of EBC analysis can be based.

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APPENDIX 1 – List of manufacturers

BMG LABTECH GmbH, Hanns-Martin-Schleyer-Str. 10, 77656 Offenurg, Germany

BOC Gases, The Priestley Centre, 10 Priestley Road, Surrey Research Park, Guildford, Surrey, GU2 7XY, UK

B&Q, Torrance House, Erskine, Renfrewshire, PA8 6AT, UK

Cranlea and Company, The Sandpits, Acacia Road, Bournville, Birmingham, B30 2AH, UK

Hanna Instruments Ltd., Eden Way, Pages Industrial Park, Leighton Buzzard, Bedfordshire, LU7 4AD, UK

Microsoft Corporation, One Microsoft Way, Richmond, WA 98052- USA

Minitab Inc, Minitab Headquarters , 3801 Enterprise Drive, State College, PA 16801, USA

Sigma-Aldrich Company Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT, UK

APPENDIX 2 – List of abbreviations

am	In the morning
ANOVA	Analysis of Variance
ARDS	Acute respiratory distress syndrome
ATS/ERS	American Thoracic Society/European Respiratory Society
BAL	Broncho-alveolar lavage
BALF	Broncho-alveolar lining fluid
Bpm	Breaths per minute
°C	Degree Celcius
C _{dyn}	Dynamic lung compliance
cm	Centrimetre
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variation
EB	Exhaled breath
EBC	Exhaled breath condensate
EHV	Equine Herpes Virus
ERhV	Equine Rhino Virus
HRP	Horse radish Peroxidase
IAD	Inflammatory airway disease
IFN	Interferon
IL	Interleukin
IU/L	International Unit per Litre
L	Litre
LAI	Lower airway inflammation
LDL	Lower detection limit
LQL	Lower quantification limit
M	Molar
min	Minute
MIP-2	Macrophages inflammatory protein 2
ml	Millilitre
NF	New Fores
nm	Nanometre
P _a CO ₂	Partial pressure of carbon dioxyde in arterial blood
P _a O ₂	Partial pressure of oxygen in arterial blood
pm	In the afternoon
PP	Polypropylene
RAO	Recurrent airway obstruction
RONs	Reactive oxygen and nitrogen species
R _L	Pulmonary resistance
SD	Standard deviation
SPA-RAO	Summer pasture associated – recurrent airway obstruction
Spp.	Species
TA	Tracheal aspirate
TB	Thoroughbred

TMB	3,3',5,5'- tetramethylbenzidine
TNF	Tumor necrosis factor
WB	Warmblood
X	Cross
y.o.	Years old
ΔPpl_{max}	Maximal change in pleural pressure
μM	Micromolar
μL	Microlitre

APPENDIX 3 – Figures of equipment for EBC collection



Figure 1: the facemask used in the study was built from a plastic muzzle adapted with fibreglass and resin. Two openings were used to attach one-way valves

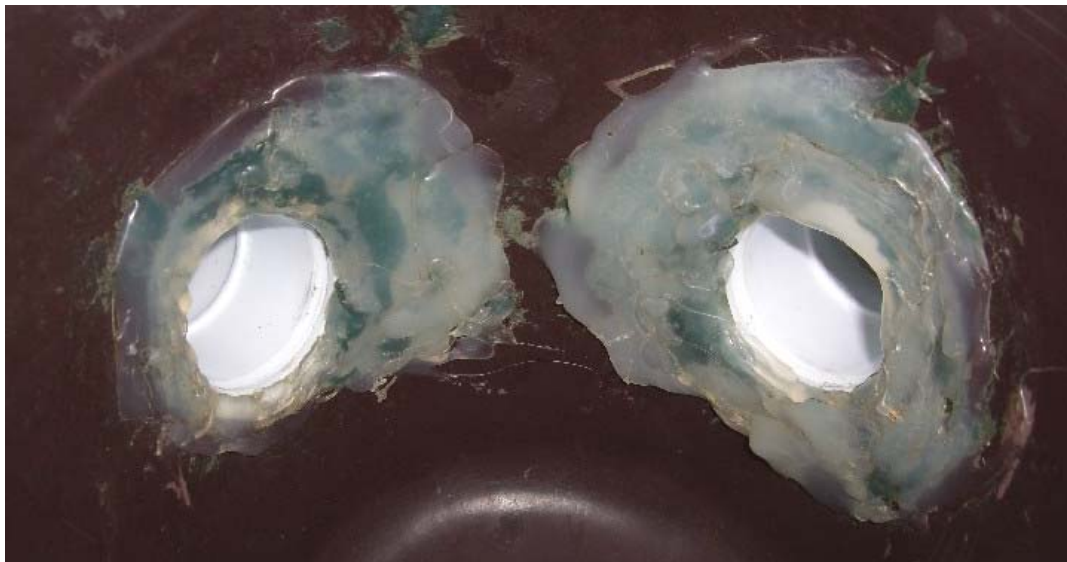


Figure 2: inner view of the mask where two holes were left open and modelled with fibreglass and artificial resin to fit the attachment of the one-way valves



Figure 3: A rubber ring was used to establish an air-tight connection between each one-way valve and the mask



Figure 4: the rubber shroud sealed the mask on the muzzle of the horse creating a closed unidirectional circuit preventing contamination of the exhaled breath sample with ambient air and permitting generation of negative pressures necessary to activate the one-way valves.



Figure 5: One-way valves used in the study. Two valves were connected to the mask. Each valve has an internal diameter of 3.5cm and ensure a unidirectional flow of air during respiration by the horse



Figure 6: A) The connection of the face mask to the condensation chamber was obtained through flexible rubber/plastic tubes; B) The device is used in the standing non-sedated horse



Figure 7: The condensation chamber has a one way valve (A) at the end of the system to isolate of from the environmental air. The PP chamber (B) in immersed in the ethanol/liquid nitrogen slurry

APPENDIX 4 – Figures of laboratory analysis of EBC



Figure 1: A) The Horse Radish Peroxidase (HRP) was available as a powder and was reconstituted as a solution by adding 1.36ml of distilled water for every mg of powder; B) The TMB (3,3',5,5'-tetramethylbenzidine) used was available in 1mg tablets and was prepared in solution mixing 1mg of TMB with 0.5ml of Acetone, 0.5ml of methanol and 9ml of 0.42M citrate buffer solution (pH 3.84)

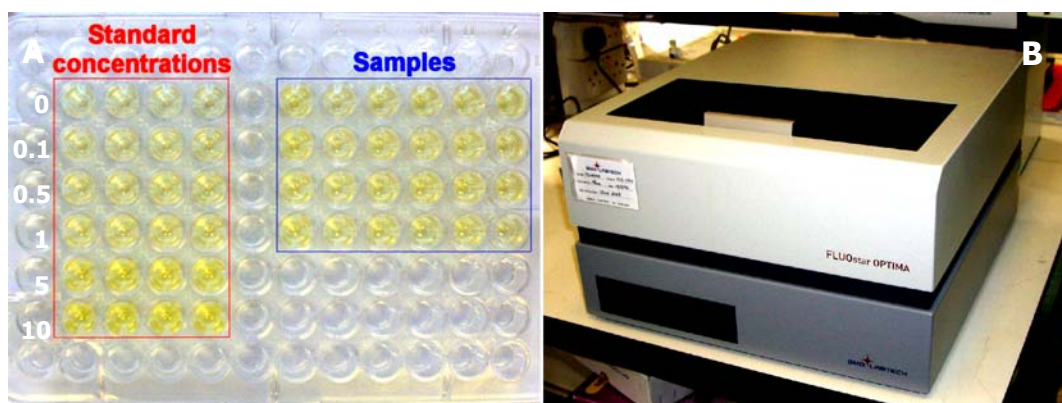


Figure 2: A) Microplate ready for analysis with standard concentrations (0, 0.1, 0.5, 1, 5 and 10µM) on the left and samples of EBC on the right. B) The spectrophotometer read the absorbance of each well (FLUOstar OPTIMA, BMG labtech, UK) at the wavelength of 450nm

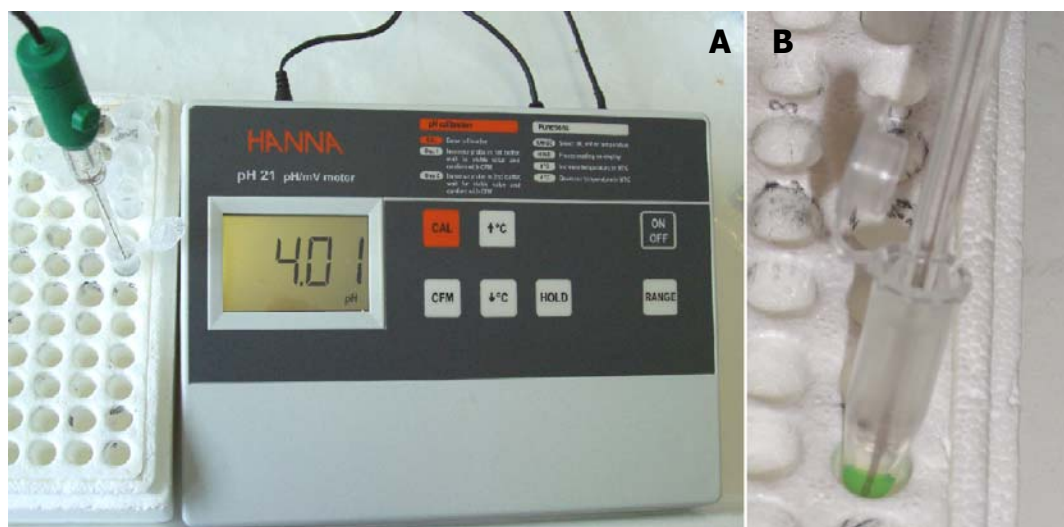


Figure 3: A) Electrochemical bench pHmeter and B) electrode (6mm diameter) used to measure the pH of exhaled breath condensate in sample as little as 0.3ml