Biochemical and Proteomic Investigation of Bovine Nasal Secretion

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Ph.D)

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University of Glasgow

November 2014

Abstract

The principal aims of the work presented in this thesis were to investigate the biochemical properties and protein compositions of nasal secretion (NS) from healthy cattle and to document changes in the protein expression in NS from diseased and vaccinated cattle using advanced proteomic methods.

Bovine respiratory disease (BRD) is the principal source of economic loss for the cattle industry throughout the world. Even though it has been studied extensively, it still remains the number one cause of disease and death in cattle. Although the etiopathogenesis of BRD is multifactorial and complex, all of the pathogens have the common route of infection which is intranasal. Thus, NS is potentially a valuable source of biological sample in the detection of biomarkers for BRD.

Therefore, a method for the collection of substantial volumes of NS from cattle was developed in this study to establish a reference range of analytes that are present in the NS of healthy cattle. Biochemical profiles of NS from a group of 38 healthy Holstein-Friesian cows revealed high alkaline phosphatase (AP) activity of up to 2392 IU/L which is 15.7 fold higher compared to AP activity in serum reference ranges.

This study further investigated the source of the high AP activity in bovine NS. Histochemical analysis using AP specific staining confirmed the localization of the AP enzyme activity to epithelial cells and serous glands of the nasal mucosa. Advanced molecular methods were used to determine the characterization of nasal AP. The analysis at mRNA levels from nasal mucosa by endpoint RT-PCR and PCR product sequencing confirmed that the AP was locally produced and is identical at the nucleotide level to the non-specific AP splice variant gene (*ALPL*) found in bovine liver, bone and kidney. Investigation using isoelectric focussing (IEF) confirmed that AP was produced locally at a high level in nasal epithelium demonstrating that novel AP isoenzymes in NS had pIs in the same range as those of the nasal mucosa extracts (pH 4.8-6.2) but were clearly different from the extracts of other tissues. The differences in IEF migration of the AP extracts is likely to be due to post translational modifications (PTMs) such as glycosylation or phosphorylation which would be areas worthy of future research.

Preliminary proteomic investigation of NS from healthy cattle using 1D gel electrophoresis, 2D gel electrophoresis and ESI-MS/MS analysis putatively identified 10 major proteins in NS consisting of 7 vascular proteins and other glandular and cellular

proteins such as lactoferrin, an anti-bacterial protein commonly present in mucosal secretions, odorant binding protein known to have a role in scent recognition and glutathione-S-transferase, an enzyme capable of detoxifying noxious compounds.

The final objective was therefore to compare the nasal proteins from healthy, disease and vaccinated group of animals using 2D difference gel electrophoresis (DiGE). The experimental model used for this investigation was an immunisation study against bovine malignant catarrhal fever (MCF). It was concluded that quantitative proteomics technology such as DiGE identified and measured changes in nasal protein expression in response to MCF and following vaccine protection.

In conclusion, this research has contributed to the scientific knowledge regarding the biochemical properties and protein compositions that is present in bovine NS. In addition, the research also explored the use of proteomic technologies as a novel tool to analyse protein expression and identify possible biomarkers in NS. This thesis is an important step forward for a better understanding of bovine NS, and thus provides a basis for future studies involving bovine NS by way of providing reference data and alternative source of biological sample.

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Acknowledgements

In the name of Allah SWT, the Most Gracious and the Most Merciful, I offer You my humble thanks and gratitude for giving me the strength in completing this thesis.

I am forever grateful to many individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this thesis. I would like to begin by acknowledging the Ministry of Education Malaysia and Universiti Sultan Zainal Abidin for providing the financial support that allowed this PhD journey to become a reality.

I am deeply indebted to my principal supervisor Professor David Eckersall for his fundamental role in my doctoral work. David provided me with every bit of guidance, assistance, and expertise that I needed throughout my PhD; then, during the writing up of my thesis, David gave me the freedom to work at my own pace, at the same time continuing to contribute valuable feedback, advice, and encouragement. I quite simply cannot imagine a better supervisor. My deepest gratitude also goes to Professor Nick Jonsson, my co-supervisor who has guided me throughout this journey. His excellent advice and constructive criticism in both experimental and thesis works deserve special recognition.

I would like to acknowledge the inspirational guidance that I have received from Dr. Mark McLaughlin on molecular biology techniques. Mark has been most generous in sharing his scientific knowledge and giving his time to assist me in many ways throughout my study. I am also extremely grateful to Dr. Paul Montague for his help in developing the alkaline phosphatase primer for this study.

Special thanks to Professor Sarah Cleaveland for sharing with me her method in collecting bovine nasal secretion and Dr. George Russell for providing me the bovine nasal secretion samples used in my DiGE experiments. Without their help, this project would not have been possible. I am also extremely grateful to the stockperson at Cochno Farm especially Mr. Ian Cordner and Mr. Robert Gault for assisting me in collecting nasal secretion samples as well as to Mr. Richard Irvine and Mr. Michael Mcguigan from the post mortem unit for providing me plenty of tissue samples throughout my study. Without their help, my work would not have been completed.

In the various laboratories, I have known many important people being most generous in sharing their knowledge with me and giving me a helping hand. I wish to express my

utmost gratitude to Dr. Caline Koh-Tan and Mr. Iain Macmillan for their patience and constant assistance in my study especially with the histochemistry. I am also extremely grateful to Dr. Richard Burchmore, Mr. Alan Scott and Miss Suzanne Eadie for their knowledge and technical assistance in my proteomic works. Special thanks to Dr. Rozaihan Mansor, Miss Mary Waterston and Miss Eilidh McCulloch for their continuous help in my laboratory works. Many thanks also to Mr. Chris McComb for sharing me his knowledge on gene sequencing. Not to forget my special thanks to Mr. James Harvie for his continuous help in biochemical analysis and enthusiastically answering all my queries with his thick Scottish accent.

I have been very privileged to get to know many other great people who became friends over the last few years. I am extremely grateful to my fellow colleagues Mark Braceland, Emily O'Reilly and Funmilola Thomas for their sharing of knowledge, as well as the much help and support that they have given to me. It has been a pleasure to work with you guys! I would also like to take this opportunity to thank all my friends and family for their understanding, love and encouragement to me throughout my time here in Glasgow.

Finally, my deepest gratitude goes to Siti Mariam Zainal Ariffin for always being there for me through thick and thin. Many thanks for your constant help, understanding, support and encouragement. Without you giving me the much needed tears of laughter, I could never have completed this journey with a smiling face.

Last but not least, please accept my sincere apology to everyone that I have not mentioned personally in this thesis.

M. Faizal Ghazali, November 2014

Author's Declaration

This thesis and work contained within it, was conducted from January 2011 to November 2014 under the supervision of Professor P. David Eckersall and Professor Nicholas N. Jonsson at the School of Veterinary Medicine, University of Glasgow. All results presented, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

M. Faizal Ghazali, November 2014

Dedication

This thesis is lovingly dedicated to my parents, Haji Ghazali Mhd Sharif and Hajah Ayesha Ngim Abdullah. Their support, encouragement, and constant love have sustained me throughout my life.

List of Publications and Presentations

Some of the work contained in this thesis has been the subject of the following publications or presentations:

Papers

MF Ghazali, HHC Koh-Tan, M McLaughlin, P Montague, NN Jonsson and PD Eckersall 'Alkaline phosphatase in nasal secretion of cattle: biochemical and molecular characterisation' *BMC Veterinary Research.* **2014**, 10 (1), 204 **(in Appendix A).**

Conference proceedings

2014 **ISACP 2014. 16th Biennial Congress of the International Society for Animal Clinical Pathology**
'Proteome analysis of nasal secretion from healthy, malignant catarrhal fever (MCF) challenged and vaccinated cattle', *Copenhagen, Denmark*

2014 **68th Annual Conference. Association Veterinary Teachers Research Workers, (AVTRW)**
'Comparative Proteome analysis of nasal secretion from healthy, malignant catarrhal fever (MCF) challenged and vaccinated cattle', *Nottingham, UK*

2013 **Moredun Research Institute and University of Glasgow Animal Health Workshop**
'Biochemical and Proteomic Investigation of Bovine Nasal Secretion', *Midlothian, UK*

2013 **3rd Meeting. COST Action on Farm Animal Proteomics**
'Isoelectric Focusing in Characterization of Alkaline Phosphatase Isoenzyme from Bovine Nasal Secretion and Mucosa', *Kosice, Slovakia*

2012 **British Cattle Veterinary Association 2013 Congress**
Biomarkers of Immune Function of the Bovine Nasal Mucosa', *Telford, UK*

2012 **European Proteomics Association (EuPA) & British Society for Proteome Research (BSPR) Scientific Congress**
'Proteomic and Biochemical Investigation of Bovine Nasal Secretion', *Glasgow, UK*

2012 **Glasgow Polyomics Launch Symposium**
'Biomarkers of Immune Function of the Bovine Nasal Mucosa', *Glasgow, UK*

2012 **ISACP 2012. 15th Biennial Congress of the International Society for Animal Clinical Pathology**
'Is Alkaline Phosphatase Activity in Bovine Nasal Secretion a Result of Local Synthesis?', *Ljubljana, Slovenia*

2012 **67th Annual Conference. Association Veterinary Teachers Research Workers, (AVTRW)**
'The Source of Alkaline Phosphatase Activity in Bovine Nasal Secretion is the Nasal Mucosa', *Nottingham, UK*

2012 **2nd Meeting. COST Action on Farm Animal Proteomics.**
'Biochemical and Proteomic Investigation of Bovine Nasal Secretion', *Vilamoura, Portugal*

List of Abbreviations

%	Percent
£	Pound sterling
$	Dollar
+	Plus
+ve	Positive
-	Minus
-ve	Negative
±	Plus minus
<	Less than
>	More than
α1-AGP	Alpha 1 acid glycoprotein
µg	Microgram
µg/ml	Microgram per litre
µl	Microlitre
° C	Degrees Celsius
1-DE	One-dimensional electrophoresis
2D	Two dimensional
2-DE	Two-Dimensional electrophoresis
4CN	4-chloro-1-naphthol
A	Adenosine
ACN	Acetonitrile
AlHV-1	Alcelaphines Herpesvirus 1
ALPI	Alkaline phosphatase intestinal gene
ALPL	Alkaline phosphatase liver gene
ANOVA	Analysis of variance
AP	Alkaline phosphatase
ApoA-1	Apolipoprotein A-1
APP	Acute phase protein
APR	Acute phase response
AST	Aspartate transaminase
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bp	Base pair
BSA	Bovine serum albumin
BCA	Bicinchoninic acid
BRD	Bovine respiratory disease
C	Cytosine
C	Complement
Ca^{2+}	Calcium ion
cDNA	Complementary deoxyribonucleic acid
CHAPS	3[(cholamidopropyl)dimethylammonio]-1-propane sulphonate
Cl^{-}	Chloride ion
Co.	Corporation
CRP	C-reactive protein
Cu	Copper
dH_20	Distilled water
DiGE	Difference gel elctrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidephosphate
DTT	Dithiotriethol
EC	Electrical conductivity
ELISA	Enzyme linked immunosorbent assay
Epith.	Epithelium
ESI	Electrospray ionisation
ESI-MS	Electrospray ionization mass spectrometry
g	Gram
G	Gauge

G	Guanine
g/L	Gram per liter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GGT	Gamma-glutamyl transferase
GSH	Glutathione
GST	Glutathione S-transferase
H&E	Hematoxylin and eosin
Hr	Hour
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCl	Hydrochloric acid
HDL3	High density lipoprotein
Hp	Haptoglobin
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IAP	Intestinal alkaline phosphatase
ID	Identification
IEF	Isoelectric focussing
Ig	Immunoglobulin
IL	Interleukin
Inc.	Incorporation
INF-γ	Interferon gamma
iTRAQ	Isobaric tags for relative and absolute quantitation
IPG	Immobilized pH gradient
IU/L	International unit per liter
kDa	Kilo Dalton
L	Liter
LBP	Lipopolysaccharide binding protein
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharride
M	Molar
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MCF	Malignant catarrhal fever
Mg_2Cl	Magnesium chloride
MHC	Major histocompatibility complex
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MALDI-TOF MS/MS	Matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry
MOWSE	MOecular Weight SEarch
Mud-PIT	Multidimensional protein identification
Mw	Molecular weight
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
n	Sample size
Na^{2+}	Sodium ion
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
$(NH_4)_2SO_4$	Ammonium sulphate
NH	Ammonium
NH_4OH	Ammonium hydroxide
NH_4SO4	Ammonium sulphate
NM	Nasal mucosa
No.	Number

NS	Nasal secretion
NSAP	Nasal secretion alkaline phosphatase
OBP	Odorant binding protein
OD	Optical density
OvHV-2	Ovine Herpesvirus 2
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Power of hydrogen
pI	Isoelectric point
PMF	Peptide mass fingerprint
PMN	Polymorphonuclear
pmol	Picomol
PO_4^{2-}	Phosphate ion
PTM	Post-translational modification
p-value	Probability that null hypothesis is true
RNA	Ribonucleic acid
RT	Reverse transcriptase
Sec	Second
SAA	Serum amyloid A
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption/ionization
Sp.	Species
T-TBS	Tris-buffered saline with Tween 20
SRID	Single radial immunodiffusion
TBS	Tris buffered saline
Tm	Annealing temperature
TMB	Tetra methyl benzidine
TNF-α	Tumour necrosis factor alpha
TOF	Time-of-flight
UK	United Kingdom
UV	Ultraviolet
USA	United States of America
V	Volt
vs.	Versus
v/v	By volume
w/v	By weight

Chapter 1

General Introduction

1.1 Bovine respiratory disease and its impact on the cattle industry

1.1.1 Background

Bovine respiratory disease (BRD) is a general term for respiratory disease in cattle caused by a range of factors, singly or in combination. Of cattle diseases, BRD has the greatest economic impact towards the cattle farming industry and till this date pneumonia is a leading cause of loss to the cattle industry in the United States and Europe (Griffin, 1997 and Ackermann et al., 2010). An estimated 1.9 million animals (Nicholas and Ayling, 2003 and Nicholas, 2011) are affected by BRD each year in the UK cattle industry with costs estimated at around £60 million annually (NADIS, 2007). Costs associated with BRD prevention, treatment, morbidity, and mortality have been estimated from US$13.90 (Snowder et al., 2006) to US$15.57 (Faber et al., 1999) per head. Annual losses to the US cattle industry are estimated to approach 1 billion dollars, whereas preventative and treatment costs are over 3 billion dollars annually (Griffin, 1997 and Jones and Chowdhury, 2007).

Bovine respiratory disease affects the upper respiratory tract causing inflammation of various tissues such as pharyngitis, rhinitis, tracheitis and bronchitis and/or lower respiratory tract prompting the development of pneumonia. Respiratory pathogens can cause serious outbreaks of acute pneumonia in neonatal, weaned and growing calves. Whereas chronic infection leads to debilitation, decreased performance, and the need to protect the welfare of these animals suffering from the disease often leads to the early culling of the animals. In addition, another major worry are the usage of numerous and unregulated antimicrobial drugs which subsequently leads to the growing concern on the issues of antibiotic resistance. The needs to enhance effective immune responses are needed because of the high incidence of pneumonia in cattle, ubiquity of respiratory pathogens, and the general expectation by end consumers for producers to use antibiotics less frequently.

The bovine respiratory tract immune response is sophisticated and shaped by anatomic and cellular features unique to cattle, management practices, and interactions with specific microbial pathogens. Vaccines can effectively enhance resistance to some pathogens, but not all (Kasimanickam, 2010). Until recently, more attention now has been given to early detection of BRD onset through the understanding of the host innate immune response towards disease and factors that could increase innate immune activity. In spite of the advances in managerial practices, vaccines, and clinical

treatments, pneumonia still remains a widespread problem and ways to enhance host resistance to pathogen colonization and pneumonia are much required.

1.1.2 Predisposing factor

There are a variety of factors and conditions that predispose cattle to respiratory disease. Cattle have anatomic and cellular differences from humans and other species and are managed in herds all of which increases susceptibility to microbial pathogens. In healthy animal, the upper respiratory tract is colonized by a variety of bacterial pathogens that are inhaled and replicate in the tonsillar crypts and nasal mucin (Ackermann et al., 2010). Colonization of these organisms within regions of the upper respiratory tract mucosa may occupy micronutrients and receptor sites resulting in reduced colonization by pathogens. In an old study from 1964, Collier and Rossow have reported that the bacterial isolates from trachea and lung were colonized by only commensal microflora such as Pseudomonas sp., Bacillus sp., Streptomyces sp., *Micrococcus sp.* and *Streptococcus sp.*. However, Allen et al. (1991) demonstrated the presence of *M. haemolytica*, *P. multocida*, *H. somni*, *M. bovis*, and *M. bovirhinis*, *Bacillus spp.*, Streptomycetes sp., and *Neisseria spp.* in nasopharyngeal swabs of healthy calves.

One of the most important predisposing features involving the respiratory system of the cattle is the anatomic structure of the respiratory airways. Cattle have a relatively long tracheobronchial tree that increases the amount of dead space volume in comparison to pigs, dogs and horses (Kirrchvink, 2008). The increased of dead space affects the amount of fresh oxygen that can be delivered to lung and increases the risk of alveolar hypoventilation with partial obstruction. Although the increased dead space in cattle may not affect respiratory tract immunity directly, it might allow increase in the surface area for particles deposition and increased transit time of inhaled vapours, gases and aerosolized materials.

There are several environmental factors and managerial issues that were known to interrupt the respiratory tracts immunity. These include transportation, weaning, humidity, air exchange, lighting, sounds, overcrowding, changes in social structure, precipitation, fluctuations in temperature, changes in feedstuffs, feedlot floor conditions, and other microbial agents. Minor changes to these predisposing factors could even prompting distress to the animals, affecting even the basic aspects of the immune system and subsequently predisposing them to BRD.

1.1.3 Aetiology

Bovine respiratory disease is a complex infectious disease caused by the interaction of several pathogenic microbial agents. These include viruses that have a tendency to infect immunocompromised lung which includes bovine herpesvirus-1 (BHV-1), bovine adenovirus A–D, bovine respiratory syncytial virus, bovine coronavirus, infectious bovine rhinotracheitis (IBR), bovine parainfluenza virus 3 (PI-3), bovine viral diarrhoea virus (BVDV) and bovine viral diarrhea virus 1 and 2. Many herds of cattle have been reported to have been colonized by *Mycoplasma spp.* including *bovis*, *dispar*, *bovirhinis* which involve in inhibiting the function of ciliated respiratory epithelial cells (Booker et al., 2008 and Gabinaitiene et al., 2011 and Gautier-Bouchardon et al., 2014). Upon early viral infections, toxins such as 3-methyl indole or other immunosuppressive conditions would increase replication of other bacterial pathogens such as *M. haemolytica*, *P. multocida*, *H. somni*, *A. pyogenes* and *Chlamydiaceae*. These bacteria could colonize the tonsil and at the mucous of the nasal meatus and sinuses (Ackermann et al., 2010 and Love et al., 2014). Following any stresses, these pathogenic microbes shall actively increase in replication which subsequently leads to spreading the area of colonization.

Many recent studies on BRD have focused on BVDV. These viruses are classified into genotype 1 and genotype 2 (Ridpath et al., 1994). Within the 2 genotypes, a further division into cytopathic and non-cytopathic strains is made based on the presence or absence of effects in vitro. In cattle with a history of BRD, BVDV non-cytophathic biotypes were isolated more often than cytopathic biotypes, and BVDV1 non-cytopathic biotypes were isolated more frequently than BVDV2 genotypes (Fulton et al., 2000). Moreover, BVDV1 genotypes were isolated more frequently than type 2 genotypes from necropsy of calves with fibrinous pneumonia (Fulton et al., 2003).

1.1.4 Endotoxin - Lipopolysaccharide

Pasteurellaceae comprise a large family of Gram-negative Proteobacteria. Members ranging from important pathogens such as *Manhaemia sp.*, *Pasteurella sp.* and *Haemophilus sp.* that causes BRD or live as commensals on mucosal surfaces of cattle, especially in the upper respiratory tract. Lipopolysaccharide widely known as endotoxin is the major component of the outer membrane of these Gram-negative bacteria, contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack. Lipopolysaccharide also increases the negative charge of the cell membrane and helps stabilize the overall membrane structure. It is of crucial importance to Gram-negative bacteria, which would causes

bacteriolysis if the LPS are removed. Lipopolysaccharide is a major constituent of the outer leaflet of the bacterial cell membrane and a single bacterial cell contains approximately 3.5 x 10^6 LPS molecules, which are essential for growth and stability of the bacterium (Rietschel et al., 1994).

Lipopolysaccharide typically consists of a hydrophobic domain known as lipid A, a non-repeating core oligosaccharide, and a distal polysaccharide or O-antigen. The full length of the LPS molecule is shown in Figure 1.1 (Raetz, 1990). The lipid A moiety is composed of two phosphorylated glucosamine saccharides links with at least six fatty acids. Kotani et al. (1985) and Galanos et al. (1985) have reported that lipid A is the toxic part of the molecule causing effects in vivo after injection of a chemically synthesized lipid A into rabbits and mice. Schromm et al. (2000) have shown that the two phosphate groups attaching to the saccharides are essential for the toxic effects of lipid A. Removal of even one phosphate group would result in the formation of monophosphoryl lipid A (MPLA), subsequently causing significant reduced in bioactivity of LPS (Schromm et al., 2000 and Gangloff et al., 2005).

The inner core of the LPS molecule consists of two or three 2-keto-3-deoxyoctonic acid (KDO) sugars and 3 heptose sugars. Raetz, (1990) have reported that LPS structure consisting only 1 KDO sugar and the lipid A moiety is sufficient for the bacteria to maintain growth and stability. On the other hand, the outer core compartment contains common sugars with more structural diversity than the inner core. It is composed of three sugars with one or more sugars covalently bound as side chains. The O-antigen is composed of a polymer of oligosaccharides with repeating units of three sugars. The structure of this repeating unit distinguishes a bacterial strain within a serotype, which indicates pathogenicity of the antigen.

The abundance of recent genomic data has made it possible to study LPS assembly in diverse Gram-negative bacteria, including those important pathogens that cause BRD in cattle. The most important finding in the field of endotoxin biology since 1990s has been the identification of the plasma membrane protein toll-like receptor 4 (TLR4) as the lipid A signalling receptor of animal cells which involved in the innate immunity mechanism (Beutler, 2002 and Mani et al., 2012).

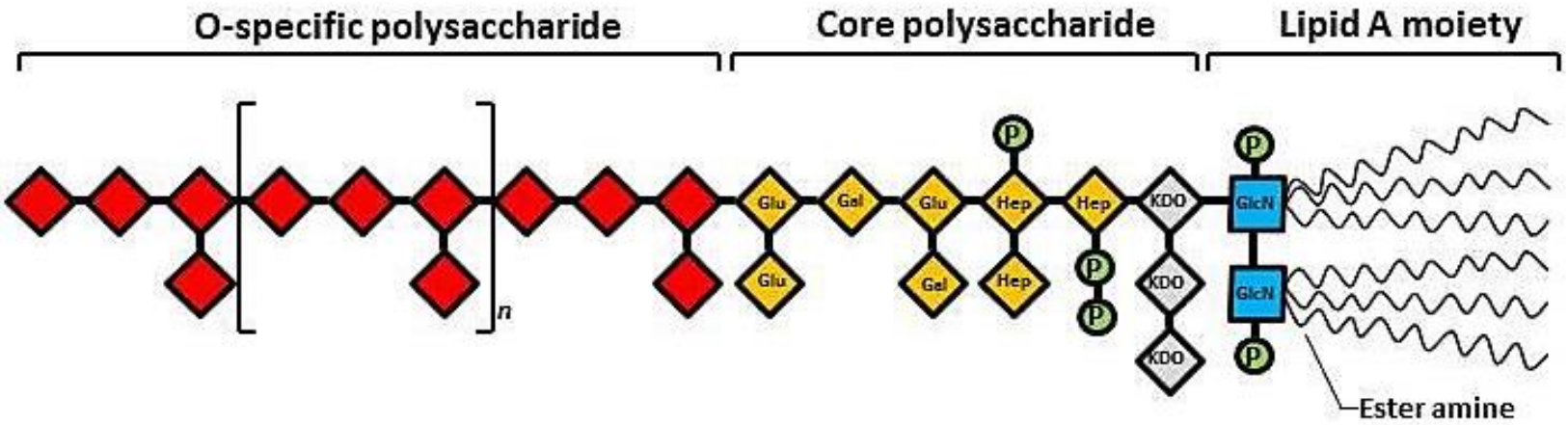


Figure 1.1: Simplified structure of LPS from Gram-negative bacteria such as *E. coli*.
Lipopolysaccharide contains a distal O-specific polysaccharide region, a core polysaccharide region divided into outer and inner core and an interior lipid A moiety through which LPS is inserted into the cell membrane. The O- polysaccharide region is highly variable and contains approximately 10 to 25 repeated units (*n*) and is made up of common hexose sugars. Outer core polysaccharide contains common hexose sugars such as glucose (Glu) and galactose (Gal), whereas inner core polysaccharide contains unusual sugar such as 3-deoxy-D-manno-octulosonic acid (KDO). Lipid A structure is explained in the text. P = phosphate; GlcN = N-acetyl glucosamine; Hep = Heptose

1.1.5 Diagnostic tests for bovine respiratory disease

Respiratory disease in the cattle industry continues to be a major cause of clinical disease, mortality, production loss, and reduced carcass quality. In addition to the initial examination of live affected animals and obtaining a history, due to the various predisposing factors and aetiologies involved in causing BRD, this disease has overlapping clinical manifestations. Hence, pathologic and laboratory investigations for the discovery of lesions and detection of the aetiology are often required in those cases for which a specific diagnosis is required. A specific diagnosis is useful to direct antimicrobial therapy, vaccination programs, and biosecurity practices.

1.1.5.1 Classical assessment

For many decades, common methods for detecting morbid cattle include visual assessment once or twice daily by the farmers or veterinarian, with cattle displaying various signs including nasal or ocular discharge, depression, lethargy, emaciated body condition, laboured breathing, or any combination of these, being removed from pens for further evaluation. Symptomatic animals with a rectal temperature ≥40°C are usually considered morbid and given treatment. Perino and Apley (1998) defined a clinical scoring system shown in Table 1.1. According to their protocol, animals with a rectal temperature of ≥40°C and a clinical score of ≥1 should receive therapeutic treatment. Due to this subjective nature of such assessment, detection of animals with BRD is not always accurate. Wittum et al. (1996) have reported that pulmonary lesions indicative of BRD were present at slaughter in 68% of steers that were not treated for BRD, whereas lesions were present in 78% of treated steers.

Likewise, Thompson et al. (2006) reported his findings in South African slaughter houses that 42.8% of all cattle had lung lesions at slaughter, and of the cattle with lung lesions at slaughter, 69.5% of them had never received treatment for BRD. Noffsinger and Locatelli (2004) have reported that the failure to detect morbid animals using current protocols for diagnosis may be related to psychological behaviour, in that when the animals perceive the cattle handlers as predators; they will mask the signs of weakness such as depression and lameness. Therefore, development and implementation of quantitative measures to detect BRD is critical, and several possible candidates will be discussed in the subsequent sections (Duff and Gaylyean, 2007).

Score	Assessment
0	Normal animal
1	Noticeable depression without apparent signs of weakness
2	Marked depression with moderate signs of weakness without a significantly altered gait
3	Severe depression with signs of weakness such as a significantly altered gait
4	Moribund and unable to rise

Table 1.1: Clinical scoring system used to assess general health of cattle.
Clinical score of ≥1 requires therapeutic intervention.

1.1.5.2 Laboratory tests

Laboratory tests are often used to confirm BRD cases however, most laboratory test procedures require time and expertise to complete, which limits their value. Field tests would be highly desirable but such tests are not widely available, often cost-prohibitive, and little data and studies are available to evaluate their efficacy. Establishing the causative agents of BRD would assist in correct treatment. A study by DeRosa et al. (2000) have shown that nasal swab cultures contained the same species of bacteria as trans-tracheal swab cultures 96% of the time and nasal swab cultures were the same with the organism causing disease in 70% of the calves lung tested in that study. A nasal swab which is easy to sample and process could routinely be performed in the field to identify the species of microbes responsible for causing bacterial pneumonia and selection of antibiotic treatments (DeRosa et al., 2000), since antibiotic susceptibility usually similar between the paired isolates for most of the antibiotics used to treat BRD.

Isolation of bacteria from cases of respiratory disease can be done from nasal, nasopharyngeal, or tracheal swabs; trans-tracheal wash or bronchoalveolar lavage fluids (BAF); or lungs at necropsy. The use of these samples to determine the cause of pneumonia in a living animal and herd health status if from necropsy samples can provide data that can be helpful for the clinician. However, there are several drawbacks which affecting this test. Mainly, all bovine respiratory bacterial pathogens can be members of the normal upper respiratory flora, and identification of one or more of such bacteria does not necessarily indicate that an organism is the cause of the disease or lesion. Another arising issue would be when bacterial isolation from an obvious clinical case of bacterial pneumonia is negative due to the usage of antimicrobial therapy.

The use of nasal, nasopharyngeal, trans-tracheal, or BAL cultures in live animals as a replacement for lung culture is less than perfect. DeRosa et al. (2000) have shown that when bacterial isolates from nasal and tracheal samples from cattle were compared, there was 96% correlation between the bacterial species recovered from the 2 sites. When isolates were genetically tested with ribotyping and antimicrobial sensitivity, only 70% of the nasal and tracheal samples were similar, indicating that multiple strains of individual bacterial species are carried in the respiratory tract of cattle. In another study by Godinho et al. (2005), *M. haemolytica* and *M. bovis* were isolated from deep nasopharyngeal swabs collected prior to euthanasia and another from the lungs collected by lung lavage and from tissue samples at necropsy were compared genetically. It was reported that there was 86% correlation between URT and LRT isolates of *M. haemolytica* and 100% correlation for *M. bovis* isolates.

Serological tests such as virus neutralization test (VNT) measures antibodies that interfere with viral infection therefore, provide a functional indication of antibody efficacy against the causative virus whereas the enzyme-linked immunosorbent assays (ELISA) results, in contrast, provide only indication that antibodies bind to the virus, and those antibodies may bind portions of the virion that are not essential for viral invasion of host cells. In addition to the assays described, toxin-neutralization assays can be used for certain bacteria that secrete toxins. Gentry and Srikumaran (1991) and Aulik et al. (2010) have reported the use of this test in detecting bovine antibodies to *M. haemolytica* leukotoxin.

The direct fluorescent antibody test (FA) is a laboratory test that uses fluorescent dye-tagged, agent-specific antibodies to detect the presence of infectious agents (Jerome, 2010). Most BRD viruses may be identified in tissues by FA testing. However, a drawback to the FA test is that samples cannot be stored or archived for long due to rapid decay of the dye activity.

Immunohistochemistry (IHC) has become an important diagnostic tool in veterinary diagnostic laboratories. Narita et al., 2000 has demonstrated the potential use of IHC for identification of bovine herpes virus in cells obtained from bovine BAL. IHC is also frequently used to detect BVDV antigen in skin biopsies (Njaa et al., 2000 and Grooms and Keilen, 2002). Furthermore, DuBois et al. (2000) have shown that IHC test seems reliable where calves recently vaccinated with modified live vaccines have not caused false positives. However, skin biopsy samples cannot be pooled, hence increasing costs of diagnosis. Larson et al. (2005) suggested using RT-PCR on pooled blood samples from 30 animals, followed by an IHC test only on animals represented in the pooled samples

that returned positive assay results. This 2-test strategy might be cost effective, but it would increase the lag time in detecting PI BVDV calves and increase the time they would be able to infect other cattle.

Viral isolation (VI) tests by diagnostic laboratories have utilized the attempt to grow virus in cell cultures from diagnostic samples (Jerome, 2010). The VI tests use susceptible cell cultures for inoculation and incubation periods. During incubation, evidence of virus replication may be observed visually under the microscope as cytopathic effects (CP). VI from blood leukocytes or serum also can be used to identify persistent infection (PI) BVDV animals. However, although the time to complete these tests varies among laboratories, generally the process could take more than 10 days to provide a definitive result. This is because for initial incubation usually is takes 7 days, and if no CP or antigen is detected, a second incubation is used. A downside to VI in cell cultures is that 2 passages are used, taking at least 2 weeks before a test is called negative. Moreover, not all viruses show cytopathology, such as non-cytopathic BVDV, hence other detection methods are used to verify the virus presence such as antigen detection by fluorescent antibody, ELISA, or IHC. Thus, there is growing use of molecular diagnostic tests such as polymerase chain reaction (PCR).

1.1.5.3 Molecular diagnostics and others

One of the most common uses of molecular technology in microbiology is for detection of pathogen DNA or RNA, identification of bacterial genera, species and subspecies as well as viral genotyping (Veir and Lapin, 2010). These technologies allow laboratories to rapidly identify bacteria without the requirements of additional time consuming biochemical tests. In the case of *P. multocida*, capsular type of A, B, D, E, or F can be determined without cumbersome enzymatic digestion techniques (Shayegh et al., 2009; Stahel et al., 2009 and Taylor et al., 2010). Similarly, molecular techniques are used for identifying BVDV types 1 and 2 (Letellier and Kerkhofs, 2003 and Kosinova et al., 2007).

In a conventional PCR, a known genetic region is amplified in a thermo cycler using polymerase to produce an amplified segment of nucleic acid. Those products are then compared to known positive controls using gel electrophoresis or sequenced and compared to published sequence for the specific agent. For RNA viruses, a reverse transcriptase enzyme reaction is required. The PCR product is directly visualized using agarose gel electrophoresis with dyes such as ethidium bromide. These gel-based PCR assays are qualitative, indicating only presence or absence of visualized product of the amplification.

For the last decade, real-time PCR has largely taken over molecular diagnostics, including molecular tests used in veterinary diagnostic laboratories. The most common sequence-specific oligonucleotide probe format used in diagnostic real-time PCR is the dual labelled Taq Man probe consisting of a fluorescent reporter dye coupled at the 59-end and a quenching dye at the 39-end (Jerome, 2010). When the probe is intact, the close proximity of the quenching dye prevents the emission of the fluorescent dye. However, during PCR primer extension the DNA polymerase enzyme digests any bound Taq Man probe, separating the 2 dyes. The reporter dye is no longer suppressed by the quencher dye and may now emit a fluorescent signal. The principle behind the quantitative real-time PCR (qPCR) is that during thermo cycling PCR amplification will begin sooner in specimens containing a higher infectious agent nucleic acid load compared to a specimen with a lower infectious agent load.

Schaefer et al. (2005) suggested that infrared thermography might be valuable for detection of BRD. Similarly, the use of radio frequency implants containing temperature probes may allow for early detection of diseases that elevate body temperature (Reid and Dahl, 2005). Though the studies on these 2 methods have not been verified for the accuracy in separating healthy and morbid animals, if successful the use of technological approaches might assist in the early detection of BRD cases.

Pathologic and laboratory investigations are necessary in situations in which a specific diagnosis or identification of the cause is required. Laboratory analysis of clinical samples can be a highly effective means of refining the clinical diagnosis in situations where the benefits of diagnosis outweigh the costs of acquiring and analysing blood, NS, trans-tracheal wash, bronchoalveolar lavage or lung biopsy samples. Elements of each diagnostic approach may hold merit in evaluating BRD in cattle, and the diagnostic path will vary depending on the goals of the farmer and veterinarian. The benefits and limitations of all the diagnostic tests mentioned in this chapter is summarize in Table 1.2 (Fulton and Confer, 2012).

Test	Use	Positive	Negative
Serology	Antibody detection	Detect vaccine responses and past infections.	Titres do not necessarily infer resistance and are not able to differentiate vaccine-induced antibodies from infection-acquired antibodies.
Culture — nasal, nasopharynx, trachea, BAL	Detect bacteria and viruses	Demonstrate the presence of colonization or active infection.	Positive culture does not necessarily mean lung infection or causative for disease. Times for results to be obtained are days to weeks.
Culture — lung lesion	Detect bacteria and viruses	Require active replication of the agent in the tissue at time of death, so isolation usually indicates that high concentrations are in tissue. Antimicrobial resistance can be determined.	Sensitivity is not great and may miss true positives due to concurrent infections and antimicrobial therapy. Times for results to be obtained are days to week.
Immunohistochemistry — lung lesion	Detects antigen in lung lesion	One can localize the infectious agent within the lesion. Strong evidence that infectious agent is related to disease.	Sensitivity and specificity depend on available monospecific immune serum or monoclonal antibodies to specific infectious agent.
In-situ hybridization — lung lesion	Detects region of genome of agent in lesion	One can localize the infectious agent within the lesion. Strong evidence that infectious agent is related to disease.	Depends on known, pathogen-specific genomic region for development of specific oligonucleotide primers.
Single PCR — nasal, nasopharynx, trachea, BAL swabs or collection	Detects genetic material of agent in sample	Provides specific evidence that infectious agent is in or recently has been in a sample.	Cannot differentiate subclinical or incidental concurrent infection from natural exposure or vaccination. Does not always detect infectious material. Cannot determine antimicrobial resistance.
Single PCR — lung lesion from supernatant of tissue homogenate	Detects region of agent genome	Potential evidence of specific infectious agent is associated with disease.	May not represent causative infectious agent within diseased tissue or differentiate natural infection versus MLV vaccine.
Multiplex PCR — nasal, nasopharynx, tracheal, BAL swab or collection	Detects region of several agents' genomes	With a single test, potential evidence of one or more infectious agent associated with disease can be determined. Test provides more information than single PCR.	May not represent causative agents within diseased tissue or differentiate natural infection versus MLV vaccine.
Multiplex PCR — lung lesion from supernatant	Detects region of several agents' genomes	With a single test, potential evidence of 1 or With a single test, potential evidence of one or more infectious agent associated with disease can be determined. Test provides more information than single PCR.	May not represent causative agents within diseased tissue or differentiate natural infection versus MLV vaccine.

Table 1.2: Comparison of uses of diagnostic tests along with their strengths and limitation (adapted from Fulton and Confer, 2012).

1.1.6 Diagnostic biomarkers

Metabolic and elemental compounds including glucose, phosphorus, low density lipoprotein (LDL), lactate, valine, and iron are known to be the biomarkers of viral infection and disease outcome (Aich et al., 2009). Some innate immune protein molecules have potential use is assessing respiratory tract physiological activity and the severity and outcome of clinical disease.

During the initial tissue insult of the disease, a set of reactions result in the release of soluble mediators termed the acute phase response (Baumann and Gauldie, 1994). Many studies have shown that acute phase proteins (APP) are clinical markers of pneumonia severity. Several APP have been measured in cattle with BRD, including fibrinogen, haptoglobin (Hp), serum amyloid-A (SAA), α-1-acid glycoprotein, ceruloplasmin, α-2-macoglobulin, and C-reactive protein (Carter et al., 2002), and APP are altered by transportation in newly weaned calves (Arthington et al., 2003).

Haptoglobin and SAA assessment in ruminants has been recognised as a valuable marker of disease in these species. Serum amyloid-A, Hp, alpha 1-acid glycoprotein produced by liver in response to IL-1 and TNF alpha are produced by ruminant during pneumonia (Eckersall and Conner, 1988; Conner et al., 1989 and Wittum et al., 1996). Haptoglobin has even been identified in broncho-alveolar lavage fluid from calves with experimental Pasteurellosis (Katch et al., 1999), although they have concluded that the Hp in the lavage fluid was resulted from leakage from the circulation across the blood-lung barrier. Wittum et al. (1996) suggested that Hp concentration was unrelated to the severity of the case or the need for treatment in feedlot cattle however subsequent results suggest that Hp may have value for assessing morbidity (Carter et al., 2002). Wittum et al. (1996) and Carter et al. (2002) has also reported that Hp was of value in assessing treatment efficacy and Berry et al. (2004) has suggested that serum Hp concentrations may be useful in predicting the number of treatments required by calves. Serum amyloid-A and Hp can differentiate acute and chronic pneumonia and alterations in Hp and apolipoprotein A1 are associated with viral infections (Aich et al., 2009). Furthermore, Godson et al. (1996) work on APP in BRD have shown that Hp concentration ranged from undetectable in the serum of most calves prior to challenge, to greater than 1 mg/ml in over one-third of the calves at the height of disease. Additionally, the concentration of Hp was associated significantly with other measures of the severity of disease.

1.2 Host response to respiratory diseases

1.2.1 Immunobiology of upper respiratory tract

The URT includes the nasal mucosa, sinuses, pharynx, larynx, trachea and bronchi. Air inhaled by animal would encounter resistance to flow in the nasal cavity and induces a turbulent flow pattern (Alexandersen et al., 2003). Boyton and Openshaw (2002) have demonstrated that this phenomenon could deflect and traps aerosolized particles larger than 10 μm in the inhaled air against the nasal mucosal lining and subsequently able to be eliminated by swallowing. Other foreign particles or organism larger than 5 μm but less than 10 μm would frequently be able to escape the defences of the nasal cavity and were deposited on a ciliated epithelial area that layering from the whole nasal turbinate to the terminal bronchioles.

Epithelial layer is consisting of glandular cells that are responsible for the release of mucus when stimulated by any disturbance of the turbulence flow of secretion which capable to entrap the inhaled foreign materials (Boyton and Openshaw, 2002). Succeeding that would be the vital role of the cilia that impede the adherence and entry of foreign bodies especially pathogens into the LRT by beating in a co-ordinated mode to propel the inhaled material towards the pharynx for swallowing (Zhang and Sanderson, 2003). For microorganism to overcome these local host defences and subsequently causing tissue disruption, attaching to host epithelial cells is vital for the beginning of a disease to develop. Bischof et al. (2008) have shown that *M. mycoides* the causative agent of contagious bovine pleuropneumonia have to first attach to calf nasal epithelial cells to achieve a cytotoxicity rate of approximately 90%. Another finding by Al-Haddawi et al. (2000) also shown that *P. multocida* attaches to microvilli and the mucous layer of respiratory epithelium through its fimbriae. Following attachment, *P. multocida* and its toxic secretions would rupture the cilia membrane resulting epithelial cells to become necrosis and slough off (Al-Haddawi et al., 1999).

1.2.2 Composition of bovine nasal secretion

On the event when the functional integrity of the epithelial layer has been compromised by damage and inflammation, the immunological barrier would be unable to stop infection. Further defence mechanisms are required to prevent invasion of pathogen into the LRT thus maintaining lung sterility and the onset septicaemia.

Nasal secretion (NS) derived from the epithelial cells secrete antimicrobial peptides and proteins (AMPs) forms a vital role in stopping further microbial invasion and proliferation. The NS include IgA being the most prevalent immunoglobulin, proteases, cathelicidins, lysozyme, lactoferrin, surfactant proteins, defensins, and lactoperoxidase which produce superoxide free radicals to destroy bacteria (Ganz and Weiss, 1997). These antimicrobial properties are capable to prevent the development of respiratory diseases in cattle and their response to epithelial compromise and microbial invasion in an experimental challenge study shall be discussed in Chapter 6. A scheme summarizing the primary function of protein commonly found in mammalian NS, which also provides insight into nasal mucosa defence mechanisms is reported in Figure 1.2.

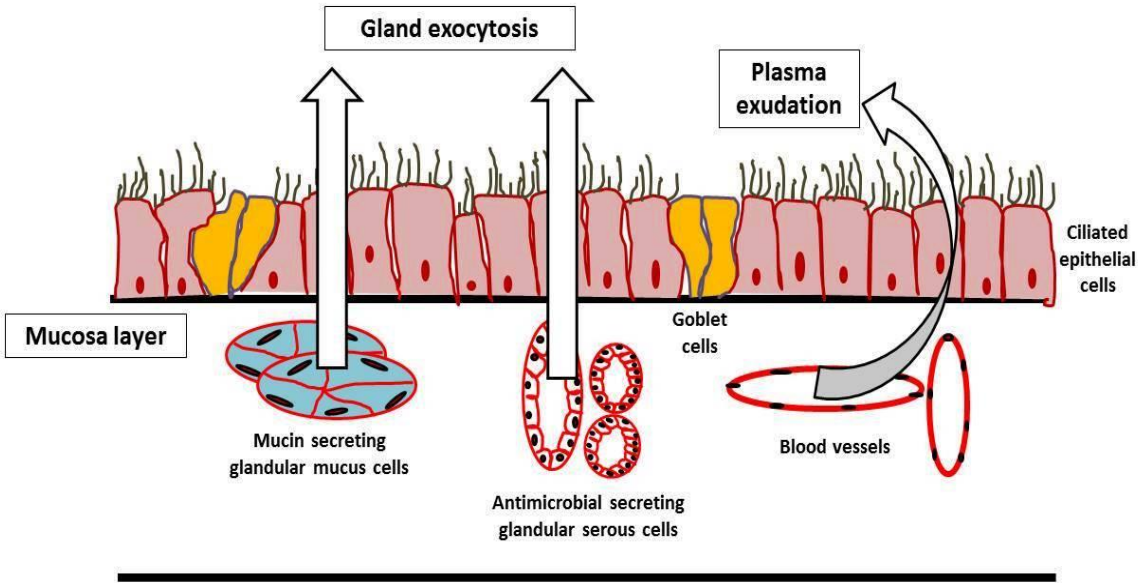


Figure 1.2: Bovine nasal mucosa and sources of secreted proteins.
Mucosa is the protective barrier between air and the tissues. Mucins are secreted by epithelial goblet and glandular mucous cells. Glandular serous cells secrets antimicrobial proteins that could bind to the mucins. Serous cells also bear the polymeric immunoglobulin receptor that transports locally synthesized IgA dimers across serous cells and exocytoses them as secretory IgA (Casado et al., 2005). Vascular permeability fills the interstitial spaces with plasma. Mucus together with the function of serous secretion traps and kills invading pathogens forming mucus raft. Ciliary action sweeps mucus raft away from nostril hence creating an innate protection in the nasal mucosa.

1.2.2.1 Immunoglobulins

In addition to providing lubrication, the submucosal gland's predominant role in the upper airways is to provide a first line of defence for the mucosa. It has long been accepted that antibody at mucous surfaces often plays a key role in preventing initial establishment and penetration of pathogens. Immunoglobulin A (IgA) masks inhaled foreign material and protect the mucosa by preventing epithelial adherence, infiltration and by augmenting toxin neutralisation. Specific immunoglobulin G (IgG) acts as an opsonin to promote the uptake of bacterial like *P. multocida* by alveolar macrophages (Reynolds, 1991).

Earlier studies conducted in respiratory tract have established that a prominent local IgA system in mammalian is located mainly in the upper reaches of the respiratory tract, with a virtual absence around the alveolar region where antibody would appear to be entirely of humoral origin. In cattle and sheep, the relative abundance of IgA presents in the NS (Mach and Pahud, 1971 and Morgan et al., 1981) in contrast to IgG presents in abundance in brochoalveolar lavages. McBride et al. (1999) have also demonstrated that immunoglobulins present in respiratory secretions with IgA are confined to the URT while IgG is more prevalent in the alveoli following *M. haemolytica* infection. The study is also consistent with the study conducted thirty years ago by Morgan et al. (1981) where IgA was shown to be the major immunoglobulin in upper respiratory tract secretions of ruminants as it is in other species studied (Morgan et al., 1981). In addition, several studies conducted on neutralizing activity in the NS of cattle following either natural or intranasal exposure to live PI-3 virus or mixed live vaccine comprising PI-3 and IBR viruses is almost exclusively associated with IgA (Morein, 1970 and Mukkur et al., 1975).

In bovine NS, IgA is present at a higher concentration than IgG. The immunoglobulins present in bovine NS, are either produced locally by cells of the lymphocyte known as the plasma cells or are derived from the blood plasma and transferred across the epithelium from the interstitial fluid compartment by selective or passive processes. Raphael et al. (1988) have demonstrated that the nasal glands in particular the serous cells process and secrete secretory IgA, the major immunoglobulin of not only the respiratory tract, but the entire mucosal immune tract. In contrast, it could be summarized that the serum proteins albumin, non-secretory IgA, and IgG enter nasal secretions through two mechanisms. The first is by increased extravasation from blood vessels into interstitial fluid and transport across the respiratory epithelium into the nasal cavity; or secondly by passage across specialized capillary fenestrae, uptake by

the submucosal glands, and transport into the glandular lumen, thus entering the nose as a component of glandular secretions (Raphael et al., 1989 and Meredith et al., 1989).

1.2.2.2 Proteases

Proteases control cellular reactions in animal at the cellular level via the cleavage of protein substrates. Metallo and serine proteases are two large families of proteases and are characterized by their catalytic type and their active site residues (Page and Di Cera, 2008). However, there is not much study conducted on the exact role of protease signalling in innate and adaptive immunity in animal.

Metalloproteases are the most diverse of the four main protease types, with more than 50 families classified to date (Visse and Nagase, 2003). Matrix metalloproteases (MMP) are a group of endopeptidase that depend on zinc as a cofactor able to degrade the extracellular matrix of lung tissue. Recent evidence has shown the role of macrophage-derived MMP (MMP-2) in the pathogenesis of pulmonary sarcoidosis (John et al., 2002). Macrophages were also found to secrete MMP-9 in a dose-dependent manner in response to a mycobacterial infection in vitro with immune-regulatory cytokines interferon-gamma (IFN-γ), IL-4 and IL-10 able to stop the bacterial-induced MMP production by reducing the amount of tumour necrosis factor-α (TNF-α) available to the macrophages (Quiding-Jarbrink et al., 2001). The specific mechanism of lung pathology caused by MMP is unknown but it is believed that MMP facilitate the migration of inflammatory cells and extracellular matrix remodelling (Obayashi, 2011 and Lee et al., 2003).

Serine proteases are widely distributed in nature and found in all kingdoms of cellular life as well as many viral genomes. They control a wide variety of physiological and pathological processes in animal and human, including tissue remodelling and local immunity against infection (Shpacovitch et al., 2008). Regulation of proteolysis induced by these serine proteases is essential to prevent self-induced damage. Hence, there are specialized serine protease inhibitors known as serpins which are broadly distributed (Silverman et al., 2001 and Heutinck et al., 2010). Serpins play a critical role in T lymphocyte mediated immunity. However intracellular pathogens may use serpins to inhibit the same proteases cell in order to escape T cell immunity (Ashton-Rickardt, 2013). However, the evolution of proteases appears to be advantageous to pathoges as it enables them to avoid destruction and opsonisation of IgA and IgG by degrading these immunoglobulins.

1.2.2.3 Cathelicidin

While humans and mice each possess a single cathelicidin, other species such as cattle and pigs express many different cathelicidins (Ramanathan et al., 2002). In cattle, Whelehan et al. (2014) have revealed seven protein-coding cathelicidin genes, with 6 of the 7 protein-coding genes were expressed in leukocytes extracted from milk of high somatic cell count (SCC) and another was expressed across several sites in the mammary gland. Although all cathelicidins are produced as a precursor consisting of an N-terminal signal peptide which is a highly conserved prosequence (Zaiou et al., 2002), it is the presence of the evolutionarily conserved prosequence that assigns an antimicrobial to the cathelicidin class (Zanetti et al., 2000).

Cathelicidin peptides generally exhibit broad-spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacteria (Zanetti, 2005). Certain cathelicidins are effective at killing common respiratory bacterial isolates of *S. aureus*, *E. faecalis* or *P. aeruginosa* that are resistant to conventional antibiotic therapy (Zanetti et al., 2002). Cathelicidins also show inhibitory activity against certain fungi like *C. albicans* (López-García et al., 2005), parasites like *C. parvum* (Giacometti at al., 2003) and certain enveloped viruses like Respiratory Syncytial Virus (RSV) causing LRT infection in human (Currie et al., 2013). Cathelicidins act as a rapid bactericidal protein where in most cases is believed to be involved in intercalation of the peptides within the bacterial membrane to disrupt membrane integrity (Gutsmann et al., 2001), although certain cathelicidins such as porcine PR-39 reach intracellular targets to block the bacterial protein synthesis (Boman et al., 1993).

The study of cathelicidin functions in human was well documented extends to other aspects of immunity and tissue repair. The only human cathelicidin (LL-37) has been shown able to bind and neutralize bacterial LPS, preventing the induction of inflammatory mediators from monocytes and endothelial cells subsequently inhibiting the development of endotoxemia in vivo (Sawa et al., 1998 and Ciornei et al., 2003). LL-37 was also shown involve in promoting wound repair by recruitment of leukocytes to the site of infection (Scott et al., 2002). Bactericidals were also effectively amplified by phagocytosis and increasing production of additional cathelicidin by the activated neutrophils (Turner et al., 1998) and mast cells (Di Nardo et al., 2003).

Cathelicidins are widely distributed within the skin and respiratory tract such as at the bronchial epithelial cells (Schrumpf et al., 2012) and other mucosal epithelial tissues (Dürr et al., 2006). Furthermore, secretions such as NS, saliva and sweat (Thienhaus et

al., 2011; Murakami et al., 2002a and Murakami et al., 2002b), are capable to serve as an important multi-functional effectors of innate immunity.

1.2.2.4 Lactoferrin and lysozyme

The major antimicrobial proteins lactoferrin and lysozyme invariably found in NS in human and animals. The iron-binding protein lactoferrin, has been demonstrated to occur in saliva, nasal secretions, tears, bronchial mucus, hepatic bile, pancreatic juice, seminal fluid, female cervical mucus and urines (Masson et al., 1966). It functions by inhibiting bacterial growth by promoting iron-restricted conditions and agglutinating the bacteria (Skerrett, 1994). These iron-binding properties are of value in the defence of epithelial surfaces against most of bacterial infections. However, an earlier study by Sparling (1983) has shown that *P. multocida* an important bacteria causing BRD in cattle are capable to overcome the effects of lactoferrin and utilises this protein as a source of iron.

Flemming and Allison in 1922 have reported the presence of lysozymes in human NS and its ability to kill bacteria by targeting their cell wall. Later, Kelly et al. (1982) have able to demonstrate that this enzyme able to specifically hydrolyse the peptidoglycan layer of the bacteria cell wall. In general, lysozymes cleaves the ß-1,4 glycosidic bond between N-acetyl-muramic acid C-1 and N-acetylglucosamine C-4 (Pahud et al., 1983). All lysozymes catalyse the same reaction hence there is a structural homology seen in different organisms such as having molecular mas of around 15 kDA and having high isoelectric point. It is well established that majority of the bacteria possess peptidoglycan. Therefore the ability of lysozyme having the bactericidal properties against these bacteria is probably due to an early destabilization of the cell wall outer membrane.

Raphael et al. (1989) have demonstrated by histochemical analysis of the nasal turbinate tissue and revealed lactoferrin and lysozyme localize within the serous cells of submucosal glands, hence providing evidence that both of these proteins are strictly glandular products within the nasal mucosa indicating that they are produced and secreted from the glands.

1.2.2.5 Surfactant proteins

Surfactant proteins are a family of collagenous carbohydrate binding proteins released by psuedostratified ciliated cells, type II and Clara cells into the airway and/or alveolar

lumen and have very potent antimicrobial and immunomodulatory roles (Wright, 1997 and Pastva et al., 2007). Surfactant protein A (SP-A) and D (SP-D) can bind and inactivate microbial agents. Grubor et al. (2006) have demonstrated that both of these proteins have a carbohydrate recognition domain (CRD) that binds to mannose residues of microbial pathogens. Once bound, the pathogen and surfactant protein complex can aggregate and be taken up by alveolar macrophages thus inhibiting adherence to epithelial lining. Once released, SP-A can activate macrophages and enhance macrophage uptake and killing of microbial pathogens. In addition, SP-A present within the alveolar lumen liquid can be taken up by the pulmonary lymphatic drainage system and enter the blood (Poulsen and McGuirk, 2009).

1.2.2.6 Defensin

Cationic peptides form a significant part of the phagocytes oxygen-independent antibacterial system. Defensins (α and ß) are an important part of the multifactorial defence against pathogens in barrier organs like the nasal mucosa (Thienhaus et al., 2011). These important cysteine-rich peptides actively involved in the innate immunity and anti-microbrial defence mechanism by way of binding to the bacterial cell membrane and neutralising the virulent effects of the bacteria (Yang et al., 2001). These peptides have been subjected to crystallisation and nuclear magnetic resonance exhibiting a distinct hydrophobic and charged region that allowing them to invade the phospholipid membrane thus assisting bacteriolysis. This ability is attributed to the 6 cysteine of each peptide that forms a circular molecule, stabilised by intramolecular bonds and creating ion-permeable channels in the membrane (Patrick and Larkin, 1995). In addition, their broad-spectrum bactericidal effects are non-specific and apply to both Gram-positive and Gram-negative bacteria (Takemura et al., 1996 and Nakajima et al., 2003) as well as modulating infections of both enveloped and non-enveloped viruses (Ding et al., 2009). These effector molecules have also been associated in the release of chemokines (IL-8) by airway epithelial cells (van Wetering et al., 2002) and degranulation of mast cells realising histamine (Befus et al., 1999) hence providing evidence for their involvement in the inflammatory response.

1.2.3 Endotoxins as activators of innate immunity

Lipopolysaccharide is the major pathogen-associated molecular pattern (PAMP) having potent pro-inflammatory properties toward many cell types (Drake et al., 1993). They are found in the cell wall of Gram-negative bacteria and in cattle as in other mammals, are recognized by the TLR4 in conjunction with the serum protein, lipopolysaccharide-

binding protein (LBP), and the CD14 co-receptor. CD14 binds to the LBP-LPS complex (Figure 1.3) and since CD14 lacks a transmembrane domain which is an accessory receptor complex (TLR4 and MD2) is required to initiate a cell signal transduction (Wright et al., 1990 and Schumann, 1992).

Lipid A is an essential part of the LPS consist of a glucosamine based phospholipid that makes up the outer monolayer of the outer membranes of most Gram-negative bacteria (Rietschel et al., 1994 and Zähringer et al., 1999). Most types of lipid A molecules are detected at pico molar levels by an ancient receptor of the innate immune system present on macrophages and endothelial cells of animals (Aderem and Ulevitch, 2000 and Medzhitov and Janeway, 2000). The receptor now identified as TLR4 (Poltorak et al. 1998, Hoshino et al., 1999), is a membrane covering protein that is distantly related to the IL1 receptor (Aderem and Ulevitch, 2000 and Medzhitov and Janeway, 2000). Lipid A activation of TLR4 in macrophages triggers the biosynthesis of diverse mediators of inflammation, such as TNF-α and IL1-ß (Beutler and Cerami, 1989) and activates the production of co-stimulatory molecules required for the adaptive immune response (Medzhitov et al., 2000). In mononuclear and endothelial cells, lipid A also stimulates tissue factor production (Drake et al., 1993 and Li et al., 1998).

In cattle and other mammals, the interaction of lipid A with TLR4 likely involves other proteins, including not only the phosphatidylinositol glycan-linked surface protein CD14 (Yang et al., 1996 and Nemchinov et al., 2006) but also the soluble accessory protein MD2 (Lizundia et al., 2008). MD2 is associated with TLR4 on the cell surface and enables TLR4 to respond to LPS, thus providing a link between the receptor and LPS signalling (Dziarski and Gupta, 2000). It is essential for the correct intracellular distribution, cell surface expression, and LPS recognition of TLR4. This may be a target for neutralizing the toxic effects of endotoxin.

In contrast, TLR2 appears to be less specific than TLR4 in that it is activated by diverse ligands, including bacterial lipoproteins and peptidoglycan fragments (Lien et al., 1999). This is though MD2 enables TLR2 to respond to non-activating LPS and lipid A, and enhances TLR2-mediated responses to Gram-negative and Gram-positive bacteria, the association with TLR2 is weaker than with TLR4 (Dziarski and Gupta, 2000). TLR6 may function together with TLR2 to recognize a subset of bacterial membrane proteins and lipopeptides (Hajjar et al., 2001). Whereas TLR3 is activated by double-stranded RNA (Alexopoulou et al., 2001), and TLR5 is activated by bacterial flagellum (Hayashi et al., 2001).

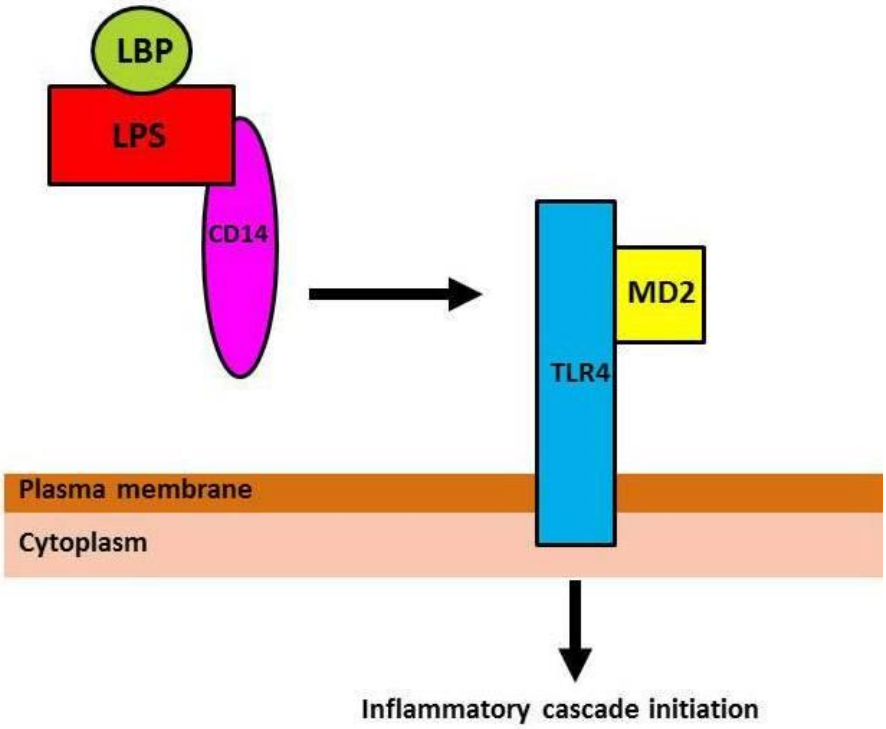


Figure 1.3: Lipopolysaccharide signalling cascade.
Signalling LPS recognition is facilitated by LBP and CD14, and is mediated by TLR4/MD2 receptor complex.

1.2.4 Systemic acute phase response

Systemic response in cattle during BRD or infections involving the respiratory normally associated with fever, pain, endocrine and metabolic alterations in serum protein concentrations characterised by the production of APP. The major APPs in cattle are mainly Haptoglobin (Hp), serum amyloid A (SAA), Ceruloplasmin (Cp) and Alpha-1 acid glycoprotein (AGP). Additionally, cytokines mainly IL-1, IL-6 and TNF-α produced by monocytes and macrophages activated by pathogens responsible for increasing the systemic acute phase response (APR).

The APR is an important feedback to avoid further destruction to the site of injury. It restricts the growth of and elimination of the pathogens as well as to remove damaged tissue and activate the process of restoring homeostasis. The APR essentially is a non-specific immune response succeeded by a specific immune response (Hirvonen et al., 1999). Under the effect of IL-1 and TNF-α and especially IL-6, hepatocytes increase the synthesis of APP which is a group of plasma glycoproteins which could increase in concentration up to 25% in response to tissue damage. The rate of increase depends on the type of APP and it is varies among species of animal. These proteins can function as

mediators, modulators, scavengers, inhibitors and immune-modulator during inflammation. Haptoglobin and SAA are two major APP related with different disease or pathological condition in cattle while Cp and AGP is a moderately responding APP. These proteins have shown to be sensitive and reliable markers for inflammation in cattle (Horadagoda et al., 1999).

1.2.4.1 Haptoglobin

The protein Hp were first documented by Bremner in 1964 showing that this protein is virtually undetectable in the plasma of healthy calves, able to reach up to 1 mg per ml during inflammation. In ruminants, there is particularly a strong Hp response to infection, with the concentration in the circulation increasing up to 100-fold or more, hence measuring of this protein could be important in assessing the health status and outcome of cattle to BRD.

In addition, Bremner (1964) has also reported that the serum Hp levels decrease during haemolysis due to an increased uptake of Hp-haemoglobin complexes by the hepatic system where they were later recycled. Haptoglobin-haemoglobin complexes were detected in 0.6% of clinically healthy cattle, however were found in most of the cows diagnosed with bacterial infection (Spooner and Miller, 1971). Haptoglobin acts as an effective antimicrobial property by rendering the iron present in haemoglobin for bacteria proliferation. Based on this ability, Eckersall et al. (1999) have shown that Hp detection is a useful diagnostic test for the detection of inflammatory disorders in animals.

1.2.4.2 Serum amyloid A

Serum amyloid A is a precursor of amyloid A with a molecular weight of approximately 9–14 kD, is an apoprotein and one of the important serum acute phase reactive proteins in cattle (Husebekk et al., 1988 and Takahashi et al., 2009). Boosmann et al. (1989) have shown that bovine SAA, increases 100-fold in cattle inoculated experimentally with *E. coli* LPS toxins. Other experimental studies have measured the SAA response in cattle following inoculation of pathogens associated with BRD like *M. haemolytica* (Horadagoda et al., 1993), bovine respiratory syncytial virus (BRSV) (Heegaard et al., 2000) and *P. multocida* (Hodgson et al., 2005). Serum amyloid-A was observed to be a sensitive APP that reacts faster than Hp following infections (Alsemgeest et al., 1994 and Horadagoda et al., 1994).

1.2.4.3 Alpha-1 glycoprotein

Alpha-1 glycoprotein is a highly glycosylated protein which build up by about 45% carbohydrate. This sugar is the composition of the glycan residues known to alter during an acute phase response (Fournier et al. 2000). AGP is considered to be a natural anti-inflammatory and immune-modulatory agent. It has also been suggested that AGP is required to maintain capillary permeability (Fournier et al. 2000). Concentration of AGP is known to increase in several cattle disease including pneumonia, mastitis and following subcutaneous inoculation of *M. haemolytica* in calves (Walker et al., 1994). Plasma concentrations of AGP rise in a slower rate following tissue injury in cattle in compare to other APP (Conner et al., 1988). In addition, AGP is also one of the most important drug binding proteins in plasma with important pharmacokinetic implications (Huang and Ung 2013) hence provide a way to monitor responsiveness to antibiotic often use in treatment of BRD cases. It exhibits a moderate acute phase response in most species and is more likely to be associated with chronic conditions.

1.2.4.4 Ceruloplasmin

Ceruloplasmin is a protein of the α-2 globulin fraction, a glycoprotein containing six copper (Cu) atoms and nine sialic acid (N-acetylneuraminic acid) residues per molecule (Frieden, 1980). It is a ferroxidase enzyme that is the major copper-carrying protein in the blood and plays a role in iron metabolism (Lovstad, 2006). In cattle, plasma Cp activities are dependent on liver Cu reserves, with lowered activities reflecting a depletion of these reserves to maintain Cu homeostasis (Paynter, 1982).

Ceruloplasmin has been evaluated as a marker of animal health and welfare (Skinner, 2001). Several studies in cattle indicate its diagnostic use with applications in many disease conditions (Sheldon et al., 2002, Laven et al., 2007 and Szczubial et al., 2008). The levels of Cp were measured to observe the effect of copper deficiency on response of calves to a respiratory disease challenge with live infectious bovine rhinotracheitis virus (IBRV) followed by intratracheal administration of *P. hemolytica* (Gengelbach et al., 1997). Another respiratory challenge study done on calves have shown that the concentrations of Cp in the serum increase during induced pneumonic pasteurellosis, with the highest concentrations observed 2 and 4 hours after the inoculation (Fagliari et al., 2003).

1.3 Alkaline phosphatase

1.3.1 Introduction

Alkaline phosphatase (AP; EC 3.1.3.1), has been continuously and extensively investigated in veterinary medicine for more than 70 years. Throughout that period, observations of the disease-related changes in AP activity in animal plasma and other bodily fluid provided a constant stimulus to explain the related pathology and extend the range of diagnostic applications.

The knowledge of the structure and function of AP has increased greatly in recent years. The importance of phosphate transfer in an animal biological system is reflected by the ubiquity of phosphate compounds. Phosphate containing compound covers many biologically important tasks, such as being essential intermediary metabolites, genetic materials, maintenance of homeostasis and reservoirs of biochemical energy (Westheimer, 1987). Due to the low reaction rates of phosphate ester hydrolyse, hence a catalyst is essentially required. The superfamily of enzymes called phosphatase has biochemical machinery enabling it to hydrolyse phosphoesters rapidly under mild cellular conditions.

Alkaline phosphatase hydrolyses non-specifically phosphate monoesters at alkaline pH to produce inorganic phosphate and an alcohol (Coleman and Gettins, 1983). Figure 1.4 describes the AP reaction. It catalyses the cleavage of a phosphate groups from a variety of compounds, including Ribonucleic Acid (RNA) and DNA and the artificial substrates (Wilson and Walker, 2000). Alkaline phosphatase also catalysis trans-phosphorilation of phosphate esters and hydrolysis, where in the presence of a phosphate acceptors, AP transfers phosphate to alcohol to form another phosphate monoester, thereby recycling inorganic phosphate within the living cells. Free inorganic phosphate is required for the uptake and the participation in biochemical reactions (Coleman, 1992). Interestingly, the final hydrolysis product, inorganic phosphate is also a strong competitive inhibitor of the enzyme, and when it is bound to the active site, it fills its entire volume (Stec et al., 2000). In addition, due to its catalytic efficiency, AP is also widely used in molecular biology labs, especially for manipulation of nucleic acids. During the DNA cloning activity of AP is used for removal of 5' phosphates. The use of a dephosphorylated vector is required to prevent it from reclosing on itself (self-ligation), and to favour it's insertion into a DNA fragment that has 5' phosphates still present. Also AP is widely used for the removal of 5' phosphates from DNA and RNA prior to labelling with radioactive phosphate (Wilson and Walker, 2000).

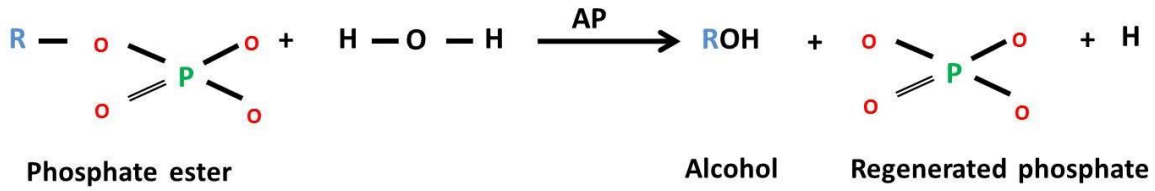


Figure 1.4: Schematic representation of the reaction mechanism of AP.

1.3.2 Regulation and distribution

As its name suggests, the tissue-nonspecific gene is widely expressed in the bovine tissues, in contrast to the localized expression of the intestinal genes or the placental genes seen in human and pig. At this moment, there are two structural genes encoding bovine AP have now been sequenced (Hua et al., 1985 and Weissig et al., 1993) and mapped to bovine chromosomes (Zimin et al., 2009). The tissue-nonspecific AP gene, expressed in osteoblasts, hepatocytes, kidney and other cells, is located at 124,938,738 <- 124,999,046 covering 99.29 centisomes on Chromosome 2. Bovine IAP as the name implies expressed by the enterocytes is encoded by a locus on chromosome 2, at 108,882,752 -> 108,932,683 covering 86.53 centisomes in legth (biocyc.org). The sequence homology between the tissue nonspecific AP and intestinal gene is 67% (Figure 1.5).

While the main features of the catalytic mechanism are conserved comparing mammalian and bacterial APs, mammalian APs have higher specific activity and Km values; have a more alkaline pH optimum display lower heat stability, membrane-bound and are inhibited by L-amino acids and peptides through an uncompetitive mechanism. These properties, however, differ noticeably among the different mammalian AP isoenzymes and are likely to reflect very different in vivo functions. Table 1.3 summarizes the nomenclature, tissue distribution and some of the functions of human and bovine AP isoenzymes. Importantly, APs have homology to a large number of other enzymes and are part of a superfamily of enzymes having some overlapping catalytic properties and substrate specificities.

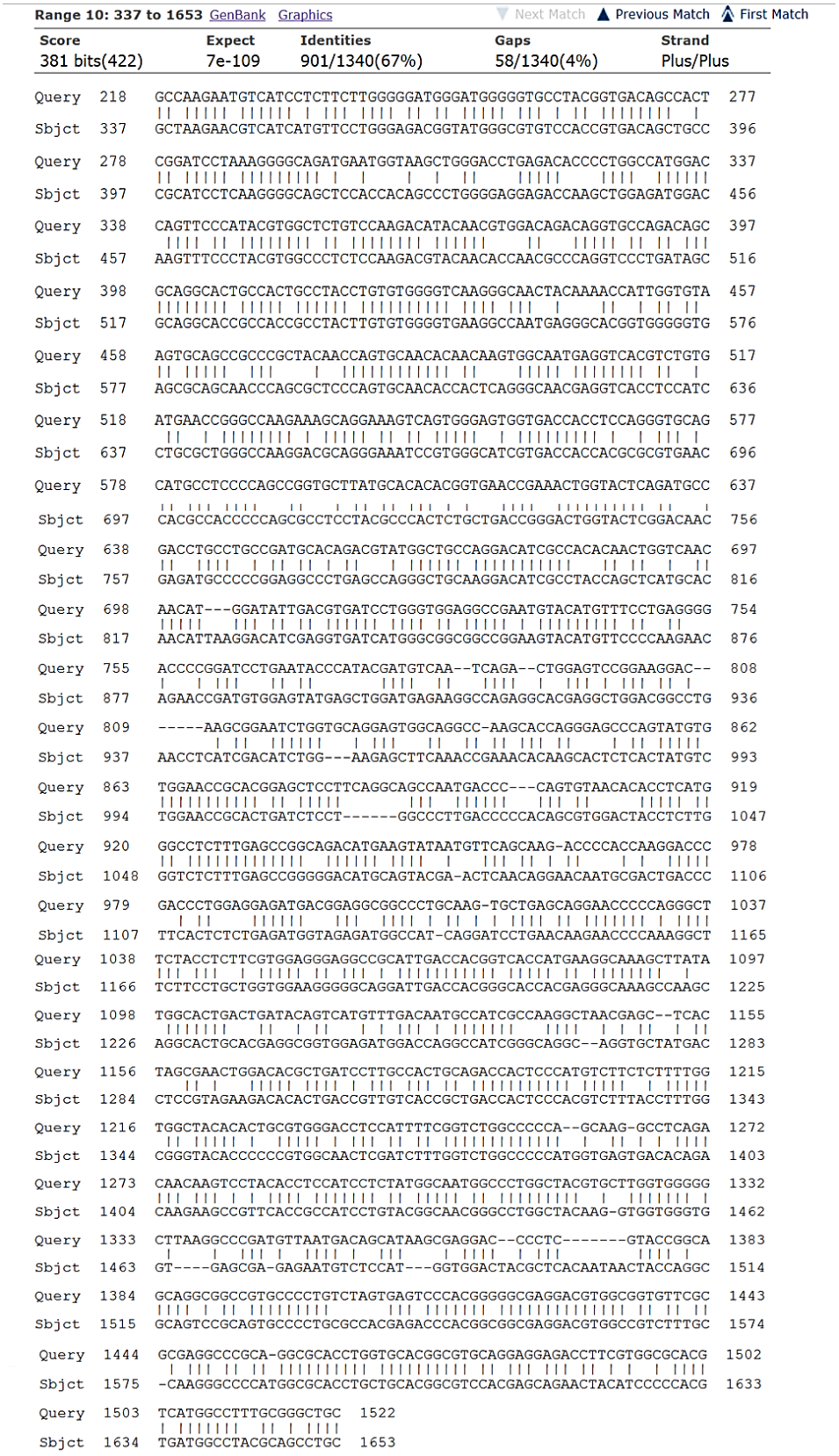


Figure 1.5: Comparison of nucleotide sequence similarity between the bovine *ALPL* gene and bovine *ALPI* gene.

	Accession numbers	Sequence length	Mass (Da)	Protein names	Common names	Tissue distribution	General function
Human genes							
ALPL	NM_000478	524[a] 447[b] 469[c]	57,305 48,909 51,045	TNAP	Alkaline phosphatase liver/bone/kidney isonzyme; Alkaline phosphatase, tissue-nonspecific isoenzyme ;TNSALP	Skeletal tissues, liver, kidney	Bone mineralization
ALPI	NM_001631	528	56,812	IAP	Intestinal-type alkaline phosphatase; IALP	Small intestine, influenced by fat feeding	Intestinal absorption
ALPP	NM_001632	535	57,954	PLAP	Placental alkaline phosphatase; PLALP	Syncytio-trophoblast, a variety of tumours	Unknown
ALPP2	NM_031313	532	57,377	GCAP	Germ cell alkaline phosphatase; GCALP	Testis, malignant trophoblast	Unknown
Cattle genes *ALPL*	NM_176858	524	57,193	TNAP	Alkaline phosphatase liver/bone/kidney isoenzyme; Alkaline phosphatase, tissue-nonspecific isoenzyme ;TNSALP	Skeletal tissues, liver, kidney	Bone mineralization, response to vitamin D,
ALPI	NM_173987	533	57,100	IAP	Intestinal-type alkaline phosphatase; IALP	Small intestine	Zinc ion binding, dephosphorylation

Table 1.3: Summary of the gene nomenclature, accession numbers, sequence length, mass, common names, tissue distribution and function for the human and cattle AP isoenzymes.
Data were tabulated from www.uniprot.org. [a-b] represents 3 different AP tissue non-specific isoenzymes in human.

1.3.3 Coding and isoenzyme

The constructed crystal structure of the placental isoenzyme in human and subsequently modelling of the three-dimensional core structures of the mammalian APs has enabled scientist to differentiate features of the animal enzymes that differ from those of the bacterial enzymes (Kim and Wyckoff, 1989). Most of the studies conducted have now accepted that AP is a homodimeric metalloenzyme with 449 amino acid residues, 30Å apart and 3 distinct metal binding sites for one magnesium and two zinc ions. The active site is located in a pocket created by the termination of a number of helices and sheets that is open to the surface (Kim and Wyckoff, 1991). Interestingly, this heterodimeric type of AP is also found in bovine pre-attachment embryos (McDougall et al., 2002) and cancer cells (Le Du and Millán, 2002).

The synthesis of this glycoprotein begins in the cytoplasm, where the translation product of AP gene (phoA) is folded into the trypsin insensitive monomeric precursor (Akiyama and Ito, 1993), which is later transported to the periplasmic space, where it spontaneously dimerises and binds metal ions (Martin et al., 1999). Disulphide bridges play an important role in correct protein folding and stability (McCammon et al., 1976). A major property of bovine APs that is important in terms of structure is the large variability in catalytic activity displayed in this species, which have 10–100-fold higher kcat values than *E. coli* AP (Murphy et al., 1995). Besman and Coleman (1985) have demonstrated the existence of two IAP isozymes in the cow intestine which is calf IAP and adult bovine IAP, by sequencing the amino termini of chromatographically purified AP fractions.

Sequence assessments (Kim and Wyckoff, 1990) between the mammalian APs and the *E. coli* AP, for which the 3 dimensional structures is known (Sowadski et al., 1985) have indicated that they conform to the basic α/ß architecture of the *E. coli* enzyme with a highly-conserved central ß-sheet. However, the eukaryotic enzymes possess highly variable loops and major insertions with no equivalents in the *E. coli* structure (Millan, 1992). These variable regions are in some instances associated with unique properties of the mammalian APs, such as uncompetitive inhibition (Hummer and Millan, 1991; Hoylaerts and Millan, 1991 and Hoylaerts et al., 1992) and binding to extracellular proteins (Van Amersfoort et al., 2003). These regions may be responsible for other novel AP isoenzyme later discovered in this study, which could have specific functions such as LPS dephosphorylation.

Alignment and comparison of amino acids sequences between bovine and human APs were given in Figure 1.6 and the percentage of similarity were tabulated in Table 1.4. The phylogenetic tree was constructed for and presented at Figure 1.7.

	Bovine TNAP	Bovine IAP	56% [a]
Human TNAP	90%	55%	
Human IAP	60%	78%	
Human PLALP	56%	74%	
Human GCAP	57%	75%	

Table 1.4: Identical percentage of amino acid sequence alignment between bovine and human APs.
[a] The homology of bovine TNAP and IAP amino acid sequence are 56%.

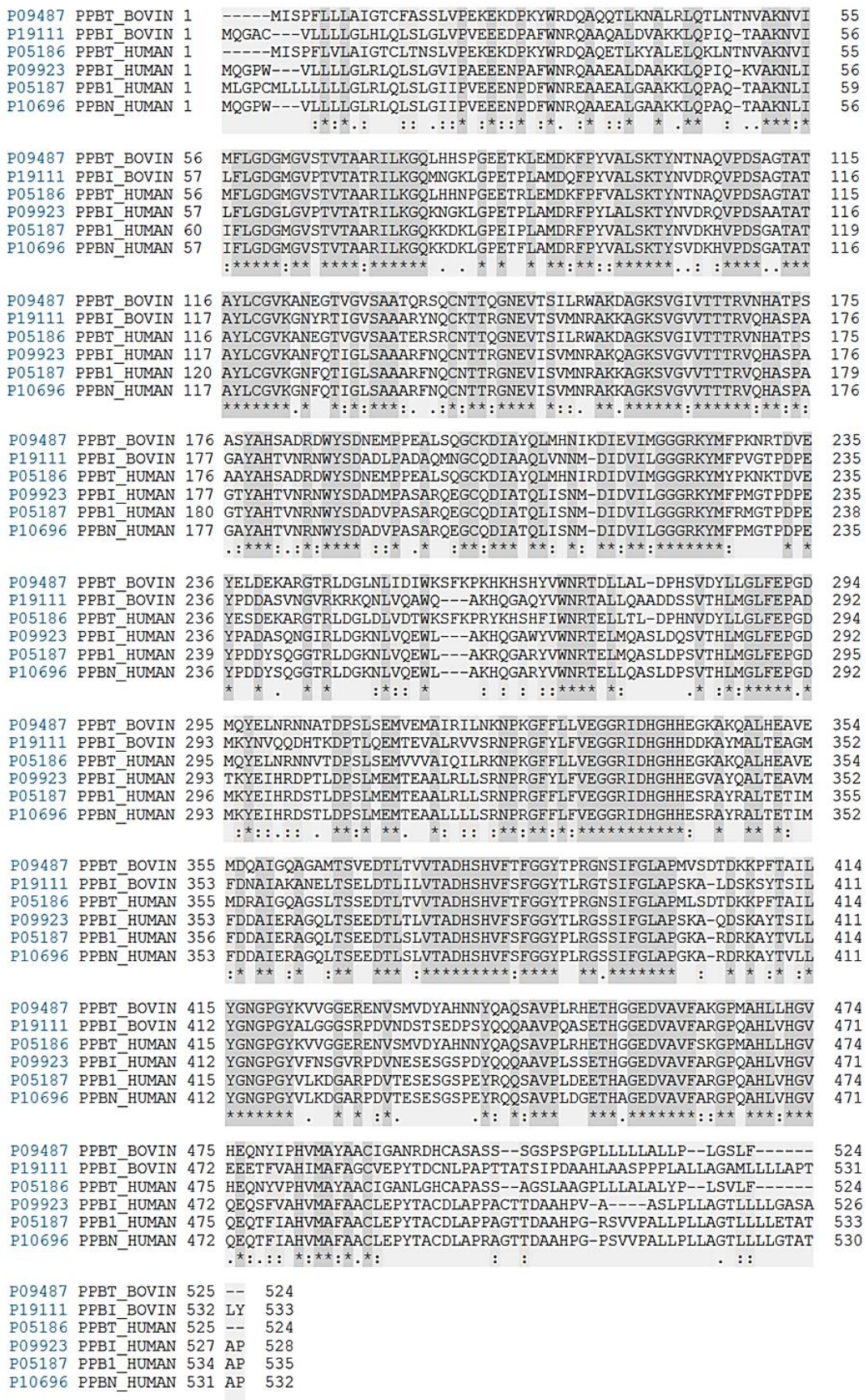


Figure 1.6: Comparison of amino acid sequences of bovine TNAP (P09487), bovine IAP (P19111), human TNAP (P05186), human IAP (P09923), human PLALP (P05187) and human GCAP (P10696).

Amino acid properties having similarity were highlighted in dark grey.

Figure 1.7: Phylogenetic tree between bovine and human APs.
Bovine TNAP (P09487), bovine IAP (P19111), human TNAP (P05186), human IAP (P09923), human PLALP (P05187) and human GCAP (P10696).

1.3.4 Alkaline phosphatase and lipopolysaccharides

AP is abundantly expressed at locations where possible endotoxin producing microbes, like *M. haemolytica* or *P. multocida*, may enter the systemic circulation via the respiratory or digestive tract. Exposure of cells that form a barrier between the host's internal milieu and the external environment to LPS result in upregulation of AP, suggesting that AP also serves a role in the natural defense system against an LPS insult (Koyama et al., 2002; Hayama et al., 2002; Koyama et al., 2004 and Vaishnava and Hooper, 2007). Furthermore, since AP catalyses the hydrolysis of phosphomonoesters and expression of AP is induced after the occurrence of LPS, it is strongly believed that this enzyme was able to detoxify LPS. Lipid A moiety contains 2 phosphate groups, and when one of the molecules might be removed by AP, this would then results in the release of inorganic phosphate (Pi) and the formation of monophosphoryl LPS (Poelstra et al., 1997a and Poelstra et al., 1997b). Monophosphoryl LPS is less effective than LPS to induce an inflammatory response, probably because it is not recognized by one of the components of the LPS cell signalling cascade.

Various studies have reported the promising effects of AP against LPS insult in a variety of animal models. A reduction in the inflammatory response induced by LPS could be observed in piglets and mice after treatment with bovine IAP (Beumer et al., 2003; van Veen et al., 2005 and Fiechter et al., 2011). Koyama et al. (2002) have shown that rats given an oral treatment of LPS have resulted in a prolonged endotoxemia when endogenous IAP was inhibited. The relative of AP reducing inflammation and preventing mortality on LPS-mediated diseases has been demonstrated in many studies involving Gram-negative bacteria insults. A study conducted in mice infected with a lethal dose of Gram-negative bacteria has shown that the mortality rate was reduced after injection of IAP derives from calves (Beumer et al., 2003). A study conducted by van Veen et al. (2005) has shown how cytokine response and neutrophil influx was seen in secondary peritonitis in mice after being attenuated by bovine IAP. Another study by Su et al., (2006) shown that sheep injected with faeces into their peritoneal to create severe peritonitis and septic shock model, have decreased IL-6 concentrations and prolonged survival time after bolus of bovine IAP was administered by intravenous.

1.4 Proteomics for the analysis of bovine nasal secretion

1.4.1 Introduction

Proteomics, the promising new "omics," has become an important complementary tool to genomics providing novel information and greater insight into veterinary diseases. Since the completion of the bovine genome, the interest of the scientific community has evolved toward understanding the bovine proteome. The genomic and proteomic data will facilitate the understanding of the functions of proteins in human and veterinary diseases and the discovery of novel drug target proteins and biomarkers of diseases. Hence, highly sensitive analytic techniques are necessary to study the complexity of biologic samples.

Wilkin et al. (1996) invented the word the term "proteome" in the mid-1990s. They have described proteome as the entire set of proteins expressed by its genome. Proteomics refers to the technical analysis of all the proteins in a given set of proteome. The application of gel-based and gel-free proteomics methods to better understand bovine response towards diseases and identification of potential disease biomarkers has strongly increased in recent years. The key to any proteomics experiment is to reduce the complexity of the sample before mass spectrometry (MS) analysis.

The application of proteomic approaches over the last decade has provided new tools for clarifying the molecular aspects of physiological states, and for understanding the aetiology and pathogenesis of many diseases. Nevertheless, proteomic studies in veterinary medicine, particularly in the bovine species, remain limited compared with human medicine. Conventional approaches to the characterization of protein changes in complex biological fluids have required the availability of species-specific antibodies for the detection and quantification of a given protein. Use of antibody-based strategies such as ELISA in biomarker discovery analyses however, limits the identification and characterization of novel candidates, as well as the detection of posttranslational modifications (PTM) of target proteins (Boehmer and Olumee-Shabon, 2005). An advantage of proteomics in biomarker discovery is the ability to detect a theoretically unlimited number of proteins in a given sample without the need for antibodies.

1.4.2 Proteome markers associated with pneumonia in cattle

Proteome based biomarkers are important for early diagnostics in veterinary medicine. Biomarker studies on farm animals usually focuses on monitoring animal health and welfare and to investigate type and state of disease, for the purpose of ensuring animal welfare as well as for monitoring quality and safety of animal products (Bendixen et al., 2011). Despite the recognized importance of NS proteins as effectors of pulmonary defence and homeostasis, as far as the author aware, no proteomic study has been done to determine how they are altered during the disease or other physiological stress response. Most of the proteomic work using biological fluid associate with the respiratory system were mostly using epithelial lining fluid (EPL). Bronchoalveolar lavage is used to sample the EPL also known as brochoalveolar lavage fluid (BALF). In human, the application of BALF to the study of lung diseases began since the mid-1970s and has contributed greatly to knowledge of local immune-inflammatory responses in normal and pathological conditions.

Proteins thought to function in the pathophysiology of several pulmonary disease states have been recently identified using proteomic analyses such as 2-DE (Magi et al., 2006) and this approach could further be used to predict markers of BRD susceptibility or identify therapeutic targets in cattle. Van der Vliet and Cross (2001) have reported that investigation of the ELF by 2-DE has revealed stress dependent alterations in several functional groups of proteins including those involved in oxidative stress such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and the APR like albumin and Hp. Other proteins that were observed in bovine EPL to have significant change during stress due to transportation were annexin A1, isocitrate dehydrogenase, fibrinogen, heme-binding protein 1 and odorant-binding protein (Mitchel et al., 2008). In another proteomic study, Mitchell et al. (2007) have examined the EPL of bovine respiratory tract and revealed that, under the influence of dexamethasone, an APR occurs with induction of adipocyte-fatty acid binding protein and OBP. Recent work from the same group using DiGE to analyse BALF collected from stressed calves caused by weaning and transportation have found that calves that later would developed pneumonia had significantly lower levels of annexin A1, annexin A2, peroxiredoxin I, calcyphosin, superoxide dismutase, macrophage capping protein and dihydrodiol dehydrogenase 3. They have concluded that the low levels of annexins A1 and A2 found to be potential biomarkers of increased the calves' susceptibility to pneumonia (Senthilkumaran et al., 2013).

1.4.3 Gel-based proteomics

1.4.3.1 Protein solubilisation

Due to the major problem of protein solubility, the majority of electrophoretic protein separations are made under denaturing conditions. Therefore, in order to take advantage of the high resolution capacity of gel electrophoresis, proteins would be completely disaggregated, reduced and solubilised to disrupt molecular interactions also to ensure separation into individual polypeptide (Gordon and Jencks, 1963).

Two types of reagents are used in 2-DE buffers to ensure protein solubility and denaturation. The first type, chaotropes such as urea or thiourea were used at multi molar concentrations, is able to unfold proteins by weakening non-covalent bonds between proteins (Gordon and Jencks, 1963). The second one is ionic detergents, in which SDS (sodium dodecyl sulfate) is the archetype. It is made of a long and flexible hydrocarbon chain linked to an ionic polar head. The detergent molecules will bind through their hydrophobic hydrocarbon tail to hydrophobic amino acids, subsequently promoting denaturing by binding favours amino acid-detergent interactions over amino acid-amino acid interactions (Abdallah et al., 2012).

1.4.3.2 One-dimensional gel electrophoresis

Gel-based proteomic is the most important and versatile method of global protein separation and quantification. One-dimensional gel electrophoresis (1-DE) became the most popular method for fast determination of protein Mw and assessing the protein purity. This widespread technique is used for many applications mainly preliminary comparison of protein composition of different samples, analysis of the number and size of polypeptide subunits, western blotting coupled to immune-detection, as a second dimension in 2-DE.

Taking advantage of both gel-based protein and gel-free peptide separation properties, 1-DE regularly coupled to subsequent analysis in liquid chromatography (LC) prior to MS. After protein separation on SDS gel, the entire gel lane is excised and divided into slices prior to the proteolytic digestion (Batycka et al., 2006). Later, peptide fractions are subjected to a second separation in LC prior to MS/MS analysis. The main aim of this technique is to ensure protein solubility during the size separation step and the reduced sample complexity prior to LC by means of exposing the harsh ionic detergent of SDS. Unfortunately, this however would further reduce the chance of identifying low abundant proteins.

1.4.3.3 Two-dimensional gel electrophoresis

Since it was first introduced by O'Farrell in 1975, two-dimensional gel electrophoresis (2-DE) has evolved at different levels and became the classical approach of protein separation and the method of choice for differential protein expression analysis (Petrak et al., 2008 and Chanthammachat et al., 2013). Two-DE separates proteins in the first dimension by IEF based on their charge at different pH values, and then in the second dimension by size and mass. This technique has an excellent resolving power, and it is now possible to visualize over 10,000 spots corresponding to over 1,000 proteins, multiple spots containing different molecular forms of the same protein, on a single 2-DE gel (Svesson et al., 2009). In addition, the use in analysis of post-translational modifications including phosphorylation and glycosylation is of paramount importance, since their alteration is frequently related with pathological states. However, moderate reproducibility and limited detection for hydrophobic proteins such as membrane proteins, low abundance proteins, proteins above or below the pore size of the gel as well as proteins beyond the pH range of the IPG strips are shortcomings of 2-DE (Chevalier, 2010).

1.4.3.4 Difference gel electrophoresis

Although no technique has a better resolving power than classical 2-DE, many endeavours were made to step forward and make it suitable to study membrane proteins (Vertommem et al., 2011), and to overcome the protein ratio errors due to low gel-to-gel reproducibility, by the use of difference gel electrophoresis (DiGE; Unlu et al., 1997).

DiGE enables protein detection at sub picomolar levels and relies on pre-electrophoretic labelling of samples with one of three spectrally resolvable fluorescent CyDyes (Cy2, Cy3, and Cy5). These dyes have an NHS-ester reactive group that covalently attaches to the ε-amino group of protein lysines via an amide linkage (Alban et al., 2003). Normally, the ratio of dye to protein is designed to ensure that the dyes are approximately covering 1-2% of the available proteins where only a single lysine per protein would be labelled. Inter gel comparability is achieved by the use of an internal standard usually by mixing of all samples in the experiment, labelled with Cy2 and co-resolved on the gels that each contains individual samples labelled with Cy3 or Cy5. Since every sample is multiplexed with an equal aliquot of the same Cy2 standard mixture, each resolved feature can be directly related to the Cy2-labelled internal standard, and ratios can be normalized to all other ratios from other samples and across different gels. This can be

done with extremely low technical variability and high statistical power (Alban et al., 2003 and Karp and Lillley, 2009).

1.4.3.5 Gel staining and protein identification

The gel must firstly be immersed in a fixation solution containing acid usually phosphoric acid or acetic acid, later with ethanol or methanol as a function of the staining protocol selected (Chevalier et al., 2007). Conventional dyes are colloidal Coomassie Blue and silver nitrate, at quite different sensitivities: 50, 10 and 0.5 ng of detectable protein spots respectively (Neuhoff et al., 1990; Mortz et al., 2001; Chevalier et al., 2007; Smejkal, 2004 and Lanne and Panfilov, 2005). Colloidal Coomassie blue or fluorescent dyes are recommended for the staining of the preparative gel, because they have good compatibility with MS (Ball and Karuso, 2007; Cong et al., 2008 and Nock et al., 2008). In contrast, Mortz et al. (2001) and Chevalier et al. (2004) have reported that silver nitrate will give poor results, even if MS compatible protocols are available. Stained protein spots of interest are excised, commonly digested with trypsin and analysed by MS, such as peptide-mass mapping by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS), and peptide sequencing by electrospray ionization tandem mass spectrometry (ESI-MS/MS) (Ong and Pandey, 2001).

1.4.4 Benefits of gel-based proteomics

1.4.4.1 Characterization of protein isoenzyme

At present, there is a growing interest by scientists in the area of identifying post-translational modifications (PTMs) and spliced gene or protein variations (Schulenberg et al., 2004 and Ahrer and Jungbauer, 2006). Today, with the option of having automatic 'in-gel' digests and identification of peptides with MS, determining protein expression in a particular proteome has become a relatively simple task. Most of the proteins are identified by either assigning them definitive protein attributes, such as peptide masses generated by MALDI-TOF mass spectrometry or with the short amino acid sequences generated by tandem MS (Chevalier, 2010). It is clear that when several spliced variants are present in a proteome, such approach for protein identification mostly characterizes peptides common to all spliced variants.

In a precedent study conducted by Hirtz et al. (2005), they have shown the used of 2-DE separation to analyse alpha-amylase isoform diversity in human saliva exist as a discrete purified protein and it is unique to its original proteome. However, the use of

electrophoretic features have allowed detail analysis of proteome separation and simultaneous hierarchical clustering of the observed peptides in the mass spectra. Consequently they have able to define several groups of isoforms present with specific sequence characteristics that potentially related with specific biological activities. In another recent study by Chevalier et al. (2009), they have shown to use 2-DE separation to analyse isoforms and polymers forms of bovine milk proteins where a combination of reducing and non-reducing steps to show proteins polymers occurring before and after heat treatment of milk. This strategy revealed numerous disulphide-mediated interactions and has proposed to analyse reduction or oxidation of milk and dairy product proteins.

1.4.4.2 Reduced gel to gel variation with DiGE

Unlu et al. first conceived DiGE in 1997 by taking advantages of the structurally protein samples, which are later co-separated on a single 2-DE gel (Viswanathan et al., 2006).The major advantage of DiGE over other gel electrophoresis based systems is that it allows the analysis of two or more protein samples simultaneously on a single 2-DE (Wheelock et al., 2006; Hrebicek et al., 2007 and Karp et al., 2008). Since the same proteins present in two different samples were pre-labelled with two different dyes commonly cyanine 3 (Cy3) and cyanine 5 (Cy5), they could be combined and separated on the same 2-DE without the loss of the relative protein abundance in the original samples. At the end of protein separation, the relative ratio of proteins in the two original samples could be readily obtained by comparing the fluorescence intensity of the same protein spots under different detection channels using a commercial fluorescence gel scanner.

Since there is only one gel is used in DiGE, and the same proteins from two different protein samples co-migrate as single spots, there is no need for superimposition of different gels, making spot comparison and protein quantitation much more convenient and consistent. This could make DiGE highly amendable for high-throughput proteomics applications, providing opportunities to detect and quantify accurately certain proteins, such as low-abundance proteins. DiGE has shown significant advantages over conventional 2-DE in a number of applications. Up to three kinds of fluorescent cyanine dyes have been employed in DiGE, namely, cyanine 2 (Cy2), Cy3, and Cy5, which allows for simultaneous analysis of up to three different protein samples in a single gel.

However, one of the problems in DiGE lies in the hydrophobicity of the cyanine dyes, which label the protein by reacting covalently with surface exposed lysine in the

protein, hence lead to the removal of multiple charges from the protein. Therefore, this decreases the solubility of the labelled protein, and in some cases may lead to protein precipitation prior to gel electrophoresis. To address this problem, minimal labelling is generally employed in when using this method. Typically the labelling reaction is optimized such that only 1-5% of total lysine in a given protein is labelled. Shaw et al. (2003) have developed a new batch of DiGE Cy3 and Cy5 dyes, which label only free cysteine in a protein by means of saturation labelling offering more sensitivity than the conventional DiGE method. The major weakness however, is that the system only labels proteins that contains free cysteine, making only a certain percentage of proteins in a proteome not labelled, causing reduced in the detection and characterization of other proteins (Arnold and Fröhlich, 2012).

1.4.4.3 Post translational modifications

Krishna and Wold, (1993) have reported that there is more than 200 post translation modifications (PTMs) of proteins that provide subtle regulatory control systems within the complex molecular machinery of cells and tissues. Two-DE gel method is suited for the detection and identification of PTMs. In most cases, the proteins are modified only at a few sites. Therefore, most digestion-derived peptides are exactly the same in the unmodified and modified forms of the proteins (Rabilloud, 2002). This ensures correct identification of the protein in the databases without requiring much peptide sequencing effort. Characterization of PTMs is crucial for biomarker discovery, because much of the regulation of the biological activity of proteins is mediated by the modification of peptide amino acid residues, including the phosphorylation of serine and threonine, and the glycosylation of asparagine, arginine, or tyrosine (Boehmer, 2011a and Boehmer, 2011b).

Detection of PTMs in 2-DE gel usually achieved by detection of altered separation parameters (pI and Mw) of that modified proteins. However, although there is always a mass alteration, it is generally too small to be detected in the SDS dimension. The only exceptions to this rule is the obvious mass increase sometimes encountered in phosphorylation and the mass decrease seen when a rather large peptide is cleaved. Therefore, the parameter leading to the detection of modifications is most often the pI. Unfortunately, this also means in turn that modifications that do not alter the pI such as cysteine alkylation or acylation will frequently remain unnoticed. Mass spectrometry can define these functional modifications that cannot be predicted from DNA or amino acid sequences alone (Mann and Jensen, 2003; Schweppe et al., 2003 and Jensen, 2004). PTMs such as phosphorylation, glycosylation, sulfation and acetylation play vital

roles in molecular interactions and enzymatic activities. Yordy and Muise-Helmenicks (2000) have reported that protein phosphorylation in human has become a major focus of interest for signal transduction pathways and therapeutic targets.

Determination of the type and precise amino acid location of a particular modification is now possible with the use of MS for both mass and ion fragment spectra. Casado (2004) have reported several novel proteomic approaches for mapping protein phosphorylation including purification of phosphorylated proteins using anti-phosphotyrosine antibodies, esterification of the amino acid residues with immobilized metal affinity chromatography (IMAC) purification, chemical derivatization of the phosphorylated proteins, application of phosphoprotein isotope- coded affinity tags to enrich low abundance phosphoproteins, identification of phosphoserine and phosphothreonine-containing peptides by MS/MS, and protein in gel digestion followed by capillary liquid chromatography (capLC-MS/MS).

1.4.5 Limitations of gel-based proteomic

1.4.5.1 Low abundance protein

Low-abundance proteins are seldom noticed or detected on traditional 2-DE maps due to the fact that large quantities of abundant soluble proteins usually would obscure their detection (Patton, 2002; Yamada et al., 2002; Greenough et al., 2004 and Ahmed and Rice, 2005). Most 2-DE based proteomic studies employ a one-extract per gel approach and the majority of proteins identified in these studies are in high abundance. These low-abundance proteins are considered to be some of the important, including regulatory proteins, signal transduction proteins and receptors. Consequently, the analysis of low abundance proteins is becoming increasingly common in cellular proteomics. The dynamic range of protein concentration can differ considerably between biological samples. However, in plasma, the predicted dynamic range of proteins is up to 12 orders of magnitude (Chevalier, 2010). Analysis of individually in parts not only provides information on protein localization, but also allows detection of protein populations that are not detectable in whole cell proteomes (Ahmed and Rice, 2005). Generally, it is require to remove abundant proteins from the sample to detect the low-abundance proteins.

The complexity of the serum and plasma proteome presents extreme analytical challenges in comprehensive analysis due to the wide variations and dynamic range of protein concentrations. Hence, a robust sample preparation usually is required as one of

the important steps in the proteome characterization. A specific depletion of high-abundant proteins from bovine serum and plasma using affinity columns is of particular interest to improve dynamic range for proteomic analysis and enable the identification of low-abundant plasma proteins (Marco-Ramell and Bassol, 2010). In addition, Smejkal and Lazarev (2005) and Hannan et al. (2009) have reported the used of narrow pIs such as (2-3 pH units) and very narrow (~1 pH unit) gradients for the first separation that enable many more proteins to be resolved. The loading capacity of narrow-range IPGs is considerably higher than broad-range IPGs, thus enabling the visualization and identification of previously unseen proteins. Sub-fractionation can be used to improve the recovery of low-abundance proteins and allow a specific separation between abundant or soluble proteins and membrane or low-abundance proteins. For example, Nissen et al. (2012) work on bovine colostrum using different fractionation techniques each have resulted in detection of unique subsets of proteins where whey production by high-speed centrifugation contributed most to detection of low abundant proteins.

1.4.5.2 High molecular weight protein

A conventional 2-DE, using SDS-PAGE as second dimension is not practical to visualize high-molecular-weight (HMw) proteins. Even with gradient acrylamide gels, it is very difficult to obtain a good separation of proteins up to 250 kDa. Alternatively, Yokoyama et al. (2009) have shown that HMw proteins and protein-complexes can be analysed using agarose gel IEF. Oh-Ishi and Maeda (2007) have also shown that agarose 2-DE is sufficiently good at separating HMM proteins with molecular masses up to 500 kDa from various diseased tissues and cells. Moreover, this method has successfully used to analyse HMw proteins previously from bovine plasma (Dahlbäck and Stenflo, 1981), dental enamel (Belcourt et al., 1983) and α-crystallin protein in bovine lens (Kramps et al., 1975 and Carver et al., 1996).

1.4.5.3 Membrane protein

The resolution of membrane proteins remains an area of considerable concern in gel-based proteomics because till recently, it is difficult or impossible to effectively resolve membrane proteins using 2-DE (Chevallet et al., 1998; Santoni et al., 2000 and Luche et al., 2003). Membrane proteins usually are poorly soluble in the detergent or chaotropic conditions in IEF, causing proteins samples to be insoluble in gel, subsequently causing them to be poorly resolved by IEF and 2-DE.

Protein fractionation in combination with the proper solubilizing reagents usually produces sample extracts that are highly enriched for membrane proteins. Steps like sequential extraction of proteins from a sample by increasing protein solubility is an effective strategy for first removing the more abundant soluble proteins and then for concentrating the less abundant and less soluble membrane proteins. Macfarlane (1989) first described the use of this cationic detergent benzyldimethyl-n-hexadecylammonium chloride in first dimension electrophoresis in view to increase the high resolution capacity for the purification of proteins from complex mixtures. Later, several studies have shown a specific 2-DE method by using the cationic detergent benzyldimethyl-n-hexadecylammonium chloride in the first and the anionic detergent SDS in the second dimension, to analyse membranes proteins in various systems (Dreger et al., 2001 and Moebius et al., 2005 and Zahedi et al., 2005).

1.4.5.4 Alkaline protein

Basic proteins were difficult to resolve on 2-DE gels. Until today, the most common commercially available pH gradients were pH 4-7 and pH 3-10 and these do not provide significant alkaline protein resolution (Wildgruber et al., 2002). As more alkaline pH range immobilized pH gradients become commercially available, resolution of proteins in IPGs up to pH 12 has been demonstrated (Wildgruber et al., 2002). Strongly alkaline proteins such as ribosomal and nuclear proteins with closely related isoelectric points (pIs) between 10.5 and 11.8 were focused to the steady state by using 3-12, 6-12 and 9-12 pI ranges (Gorg et al., 1997; Wildgruber et al., 2002 and Drews et al., 2004). Furthermore, Altland et al. (1988) and Gorg et al. (1995) have reported that for highly resolved two dimensional patterns, different optimization steps with respect to pH engineering and gel composition were necessary, such as the substitution of dimethylacrylamide (DMAA) for acrylamide and the necessity near the cathode of a paper strip soaked with DTT to provide a continuous influx of DTT to compensate for the loss of DTT. Similarly, Gorg et al. (1997) have also reported the addition of isopropanol to the IPG rehydration solution was required to suppress the reverse electro-endosmotic flow which causes highly streaky 2-DE patterns in narrow pH range for IPGs 9-12 and 10-12.

1.4.6 Proteomic and biologic discovery

Identification of biomarker is the first step towards clinical implementation (Frangogiannis, 2012). The most important role of proteomics in biologic sample analysis is the discovery and validation of biomarkers in disease. These samples include serum,

NS, cerebrospinal fluid (CSF), urine, saliva or other biological fluids. The biomarkers are generally present in low abundance, and it is necessary to work with high volumes of sample. Frantzi et al. (2014) have reported that current MS based proteomics techniques favour untargeted approaches in biomarker discovery that could result in a substantial increase of novel biomarker candidates. Petricoin et al. (2002) demonstrated that the changes in the patterns of multiple protein expression were more helpful than changes in single protein markers.These principles can be applied to bovine NS, which could be useful for the detection of biomarker in cattle having BRD or other respiratory diseases.

There were a few studies that use proteomic technology to detect potential biomarkers from human nasal lavage fluid (NLF). For example, NFL from workers suffering from occupational rhinitis were compared with 2-DE followed by MALDI-TOF before and after a 2-hour exposure to dimethylbenzylamine (DMBA). They have found palate lung and nasal epithelial clone (PLUNC) could be a potential marker for irritation. They have reported that the levels of PLUNC in the control group were lower after 2-hour exposure compared with that in epoxy workers, where the level was increased 3 to 20 fold after exposure to DMBA (Lindahl et al., 2001). Casado et al. (2002) have also shown the use of proteomics to confirm several major vascular (albumin, IgG, IgM, α-2-macroglobulin), glandular (mucin, lysozyme, lactoferrin), and epithelial (keratins) protein in human NLF. They have also concluded that a common finding was the high prevalence of lipocalin family proteins, such as PLUNC and apolipoproteins were observed in human NS samples (Casado et al., 2002). Spielman et al. (1995) and later also by Briand et al. (2002) have able to identified odorant-binding proteins OBP) in NS.

The used of proteomics to study protein expressed in human NS have proved to be important for the discovery of new biomarkers. Therefore, this technology can be a useful tool for the study of bovine NS, enabling the detection of novel biomarker in cattle suffering BRD or other respiratory diseases.

1.5 Aims of study

A non-invasive sampling technique has been the major choice of collecting biological sample from animals due to the time, cost, human safety and animal welfare concern commonly seen in experiment involving animals. In this study, the author believe that NS which produced excessively in respiratory disease animal, renders a portal through which systemic animal health could be monitored since the secretion is consist of both locally synthesized and systemic proteins that could act as novel biomarkers for BRD.

The collection of NS also provides a practical method for obtaining large volume of diagnostic samples and repeated sampling without causing stress and pain to the animals.

Hence the aims of the thesis are as follow: Firstly, to develop a method that is practical and non-invasive to collect sufficient amount of undiluted bovine NS. Secondly, to investigate the bovine NS from healthy animals and establish the normal biochemical reference data for bovine NS. Thirdly, to investigate the source of AP present in NS and subsequently characterise and compare them with those AP found in other relevant bovine tissues. Fourthly, to determine the genetic expression of NSAP and compared with the two known bovine AP gene. Fifthly, bovine NS will be screened for host protein involve in innate immunity using novel proteomic techniques. Finally, is to measure the changes of protein expression in NS from healthy, disease and vaccinated cattle using a quantitative proteomic technique.

1.6 Thesis outline

Chapter 2 begins with development and improvement on methods to collect sufficient amount of undiluted bovine NS that is practical and easy to perform. The remainder of this chapter first investigate the biochemical profiles of NS from healthy animals and then outlines the results in comparison to serum reference. Biochemical results would be use to establish a novel standard reference for future work on bovine NS. Preliminary proteomic work was also carried out to have a general assessment on the amount of protein present in NS.

Novel findings from the previous chapter direct the study to further characterized nasal AP in **Chapter 3**. In this chapter, work was focused on investigating the presence of nasal AP and understanding the function of AP in bovine NS. Studies was carried out using biochemical, histochemical, isoelectric focusing and western blot approaches in view to determine the origin of nasal AP in NS. Additionally, extraction of bovine nasal mucosa and work carried out on the tissue was also described.

Chapter 4 further describes and validates AP gene expression from nasal tissue using mixed-methods of molecular biology approaches. This chapter describes in detail the technique used such as end point polymerase chain reaction (PCR), quantitative PCR (qPCR) and gene sequencing to validate the gene as well as the results of both quantitative and qualitative analysis. The main aim of this work was to determine the

origin of nasal AP at the genome level and compare the findings with bovine liver (non-intestinal) and intestinal AP.

Chapter 5 is split into three consecutive parts, focusing mainly on using proteomic as a novel tool to investigate NS from healthy animal. In the first part, protein in NS were separated using a gel based proteomic approach mainly 2-DE which combines isoelectric focusing, separating proteins according to their isoelectric point, and then by means of SDS-PAGE, which separates them further according to their molecular mass. In the second part, the next typical steps of gel-based proteomics for both the 2-DE gels and preliminary proteomic work on 1-DE gels from Chapter 2 are spots/bands visualization and evaluation, expression analysis and finally protein identification by mass spectrometry (MS). In the last part, the chapter shall analyse and describe the nasal proteins identified and its possible function.

In **Chapter 6,** experiments were performed to compare and analyse the nasal proteins between healthy, disease and vaccinated cattle using the latest gel based proteomic approach - Difference gel electrophoresis (DiGE). Following experiments, the MS analysis results would be discussed concentrating on how the host response towards treatments and the changes occurs in NS proteins in the three different physiological conditions. The remainder of this chapter shall discuss the benefits and limitation of this method used.

Chapter 7 summarises the main findings of the research presented in this thesis and includes limitations and recommendations for future research.

Chapter 2

Biochemical profiling of healthy bovine nasal secretion

Chapter 2

2.1 Introduction

In the 1920s Fleming (1922) discovered the intrinsic antimicrobial properties of human NS and attributed them to lysozyme. Since then most of the NS studies in humans have mainly focused on the understanding of mucosal host defence against pathogens, as in common colds of either virus or bacterial origin and other respiratory diseases including hospital-associated infections (Rossen et al., 1965; Kim et al., 1983; Kaliner, 1991; Kluytmans et al., 1997; and Blackbourn et al., 1997).

An early study conducted by Long et al. (1933) at John Hopkins University had students and laboratory workers blow their nose upon a piece of non-absorbent brown paper and smearing the discharge on a glass slide, showing that macrophages, epithelial cells and polymorphonuclear leukocytes dominate the constituents of the human NS in early upper respiratory tract infections. McCormick et al. (1972) studied the acute phase of respiratory infection with NS obtained from military personnel by repeatedly flushing 5 ml of sodium chloride solution into each nostril, which was then forcibly expelled into a sterile container until a total of 100 ml was collected from both nostrils. This study revealed that the increment of IgA level during acute phase of infection derived from both serum and local production, mainly released by the IgA synthesizing plasma cells (Serum derived IgA) and glandular type epithelial cells (Secretory IgA) in the respiratory mucosa.

More recently a study described the collection of human NS by chemical stimulation with methacholine chloride using an atomizing dosimeter and vacuum-aided suction (Cole et al., 1999). They were able to identify several known antimicrobial proteins and peptides in NS such as statherins and defensins. However the major antimicrobial proteins that were present in NS were lysozymes and lactoferrin. Over many years, different techniques have been developed and used to collect this secretion. Several techniques such as nasal lavage, brush, nasal scraping or microsuction have been described for the collection of NS in human and animal models. However most of the methods have been poorly validated and findings were directly influenced by the method of collection (Watelet et al., 2004). Furthermore many of the techniques used were not applicable to an animal model.

Apart from the common techniques of diagnosing the severity of respiratory disease by taking history and clinical signs evaluation, biochemical analysis of the NS may be able to characterize the presence and severity of specific inflammatory responses, confirm the release of cytokines and mediators, detect acute phase responses and/or allow

assessment of response to treatment and vaccination. The NS closely reflects the inflammatory and immunological responses within the nasal mucosa (Watelet et al., 2004). Among the major components of the NS from human are mediators of adaptive immunity including IgA and IgG. These are both produced by plasma cells adjacent to the submucosal glands (Meredith et al., 1989). The immunoglobulins act in the mucosa to prevent the attachment and invasion of pathogenic organisms. Mediators of innate mucosal host defence are also found in NS and include substances that selectively disrupt bacterial cell walls and membranes, sequester microbial nutrients, or act as traps for microbial attachment (Cole et al., 1999). These include two major antimicrobial proteins of human NS, lysozyme and lactoferrin and other known antimicrobial proteins and peptides such as statherin, secretory phospholipase A2, and defensins, believed to contribute to the total antimicrobial properties. However there has been an increased used of electrophoresis technique to study the protein content of human NS (Cole et al., 1999).

The application of NS sample for the diagnosis of respiratory diseases in veterinary species is not common. Unlike in humans, the clinical biochemistry of NS from animal species has not been well characterised but could provide useful indicators of immune function and status. There is evidence that the nasal, bronchial and alveolar secretions have many similar properties in terms of their composition (Lindahl et al., 1995). However, unlike bronchial and alveolar secretions which are usually collected as lavage fluid with inevitable dilution, NS can easily, as shown in this chapter, be collected in an undiluted form from cattle but previously there was no suitable and practical method to collect NS from animals. Moreover, probably due to the very small volume of NS samples collected using nasal swabs, many studies conducted by using swabs mostly focus on the detection of pathogens and antimicrobial sensitivity testing (Storz et al., 1996; DeRosa et al., 2000; Seker et al., 2009; de Souza Figueiredo et al., 2010 and Klem et al., 2014). Therefore, the use of swabbing to collect NS is not sufficient for the study of host response towards diseases with tests such as biochemical and immunological analyses which usually require samples of a higher volume.

The biochemical composition of NS could provide a useful matrix for investigation and potentially provide information for the study of any respiratory diseases in cattle and other species. However before bovine NS could be assessed for use as a biological fluid to be examined for diagnosis, it is necessary to determine a baseline of the biochemical composition of the fluid in normal healthy cattle. Similarly there is a need to develop a viable technique for NS collection that is non-invasive, well standardized, reproducible

and able to provide sufficient volume for multiple investigations, allowing analysis of NS to serve as a valuable fluid for future investigation.

2.1.1 Aims of Study

The aims of the work presented in this chapter were to:

1. Provide a viable collection system for normal NS from healthy cattle with minimal invasive procedures.

2. Establish a biochemical, enzymological and immunological profile of bovine NS from healthy animals giving reference range values for this fluid.

3. Investigate the proteins present in NS using one-dimensional gel electrophoresis.

2.2 Materials and methods

2.2.1 Animals

Thirty eight Holstein-Friesian cows aged 2-5 years were convenience sampled from a herd of 90 lactating cows from University of Glasgow Cochno Farm. The animal experiments were carried out with the approval of the University of Glasgow MVLS College Ethics Committee and complied fully with the Home Office of Great Britain and Northern Ireland "Animals (Scientific Procedures) Act 1986". Clinical examination was performed to ensure the cows were clinically healthy and free from respiratory disease.

2.2.2 Bovine nasal secretion

2.2.2.1 Collection of nasal secretion

The cows were restrained in a cattle crush during the procedure. A commercially available tampon measuring 4 cm in length was inserted into one nostril and slid gently in an upwards and backwards direction about 5-8 cm deep and left in place for up to 15 min (Figure 2.1A). The tampon was then removed from the nostril by gently pulling on the attached string (Figure 2.1B) and weighed as a measure of the NS uptake before being inserted into a modified collecting tube. The cows were observed during collection and the tampon would have been removed at the first sign of distress but this was not required in any of the samples collected by this method.

2.2.2.2 Modified collecting tube

The modified collecting tube consists of a 30 ml universal tube with four holes drilled into the bottom of the tube (2 mm diameter drill bit) at the bottom, inserted in a 50 ml Falcon tube (Figure 2.2).

2.2.2.3 Extraction of nasal secretion

The modified tubes were centrifuged at 700g for 10 min at 4°C. Nasal secretion collected at the bottom of Falcon tube (Figure 2.2) were aliquoted into 1.5 ml tubes and stored at -80°C until further analysis.

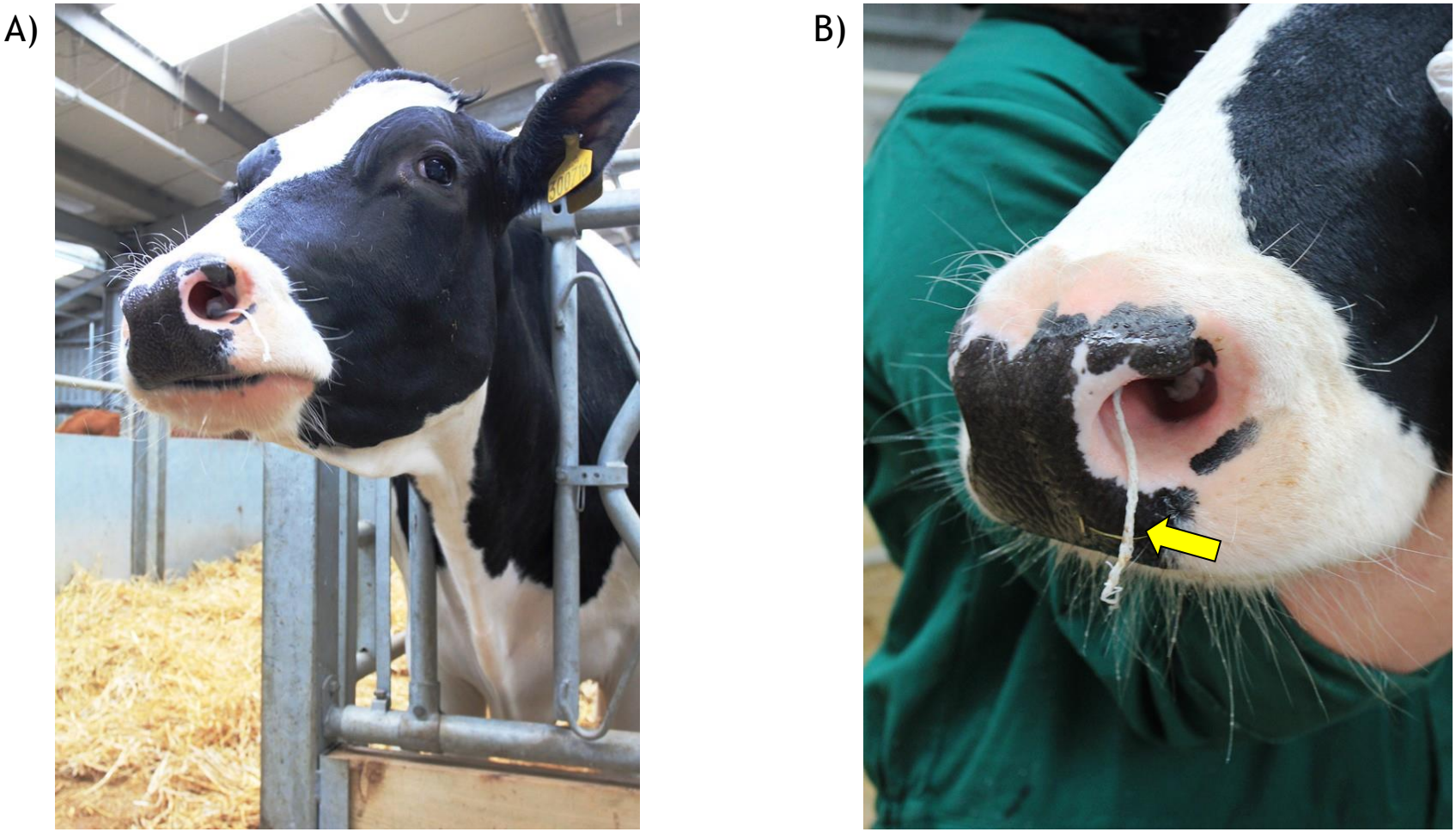


Figure 2.1: Collection of bovine NS.
A) The cows were restrained in a cattle crush during the procedure. A commercially available tampon was inserted into one nostril and slid gently in an upwards and backwards direction about 5 – 8 cm deep and then left in place for up to 15 min. B) The tampon was then removed from the nostril by gently pulling on the attached string (yellow arrow).

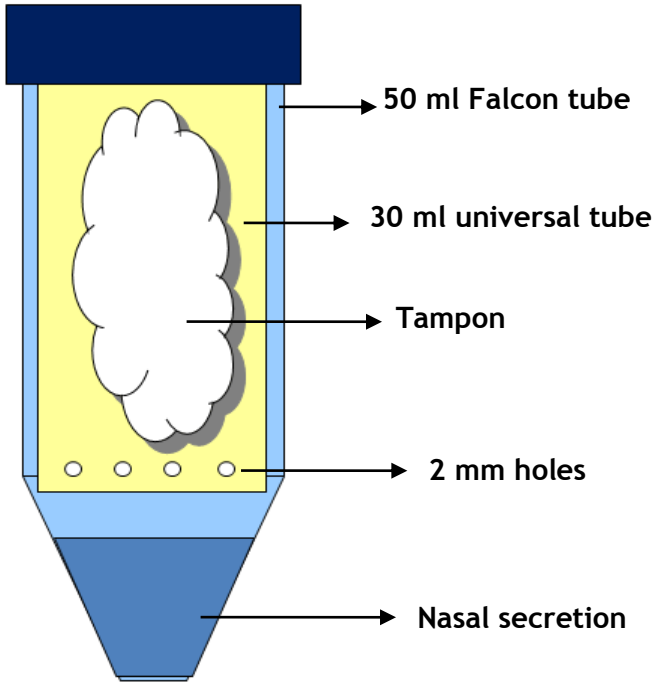


Figure 2.2: Schematic representation of a collecting tube for NS.
The saturated tampon from nostril was inserted into a modified collecting tube consisted of a 30 ml universal tube with four x 2 mm holes drilled into the bottom, inserted in a 50 ml Falcon tube. After centrifugation, NS would be collected at the bottom of Falcon tube and transferred to 1.5 ml tubes and stored at −80°C until further analysis.

2.2.3 Protein quantification

The total protein content was determined with a Bradford protein assay kit (Sigma-Chem. Co., Poole, UK) using bovine serum albumin (BSA) as protein standard. This method is a rapid and sensitive colorimetric assay for measuring total protein concentration utilizing the principle involving the binding of the dye Coomassie Brilliant Blue to protein in an acidic solution and its concomitant absorbance shift from 465 nm to 595 nm. The elevated absorbance at this wavelength reflects the increased binding of protein to Coomassie blue and in a relationship where the absorbance is proportional to the protein concentration.

Ultrapure water (10 µl) was used as a blank and a similar volume of each BSA standards; 0.25, 0.5, 1.0, 1.5, 2.0 (mg/ml) were compared to 10 µl of each sample at an appropriate dilution. Samples of NS were diluted at 1/20 and sample from tissue extracts (Chapter 3) were diluted at 1/40 in ultrapure water. Incubation of the protein mixture was conducted at RT for 5 min following addition of 250 µl Bradford reagents and mixing on a shaker for approximately 15 sec. The absorbance was read on a FLUOstar OPTIMA spectrophotometer at 595 nm (BMG Labtech, Aylesbury, UK). The details of Bradford reagents are given in (Appendix 2.1). The protein concentration was determined using a standard curve that was plotted by the spectrophotometer according to the BSA standards (absorbance versus concentration in mg/ml) and taking the dilution factor into consideration.

2.2.4 Biochemical analysis

Bovine NS collected from the 38 cows were included in this study. A total of 75 µl of NS of each was used to measure the sodium, potassium and chloride concentration using the ion selective electrode technique and a total of 10 µl was used from a sampling cup consisting of 500 µl of NS sample for measurement of other analytes (urea; creatinine; albumin; total protein; calcium; phosphate and total bilirubin). All biochemical tests (Appendix 2.2) were carried out on an Olympus A640 analyser operated by Veterinary Diagnostic Services, University of Glasgow.

2.2.5 Enzymological analysis

A total of 10 µl of NS was used to measure each of the 3 enzymological compositions (AP; AST and GGT) using an Olympus A640 analyser operated by Veterinary Diagnostic

Services, University of Glasgow using the recommended protocol of the German Society for Clinical Chemistry (Büttner, 1976).

2.2.5.1 Alkaline phosphatase

Alkaline phosphatase activity was determined by measuring the rate of conversion of p-Nitrophenyl phosphate (pNPP) to p-Nitrophenol (pNP) in the presence of magnesium ions and diethanolamine as phosphate acceptor at pH 9.8. The rate of increase in absorbance due to the formation of pNP is measured at 410/480 nm and is directly proportional to the AP activity in the sample.

Reaction principle:

$$pNPP + H_2O \xrightarrow[Mg2+]{AP} Phosphate + pNP$$

2.2.5.2 Gamma-glutamyl transferase

Gamma-glutamyl transferase (GGT) catalyses the transfer of the gamma-glutamyl group from the substrate, gamma-glutamyl-3-carboxy-4-nitroanlide to glycyglycine, yielding 5-amino-2-nitrobenzoate. The change in absorbance at 410/480 nm is due to the formation of 5-amino-2-benzoate and is directly proportional to the GGT activity in the sample.

Reaction principle:

L-γ-Glutamyl-3-carboxy-4-nitroanilide + Glycylglycine $\xrightarrow{\gamma\text{-GT}}$ L-γ-Glutamylglycylglycine + 5-amino-2-nitrobenzoate

2.2.6 Immunological analysis

The 38 NS samples collected and processed as described in section 2.2.2 were analysed for the nasal mucosal antibody contents by measuring the IgA and IgG concentrations.

2.2.6.1 Reagents and buffers

General chemicals and buffers (Appendix 2.3) were obtained from Sigma Chem. Co, Poole, UK unless otherwise stated. Double distilled water was used throughout.

2.2.6.2 Antibody

Both coating antibodies (bovine specific anti-IgA and IgG) and signal antibodies (sheep anti-bovine IgA-HRP and IgG-HRP conjugate) for ELISA quantification were obtained from Bethyl Labs, Cambridge, UK.

2.2.6.3 Immunoglobulin A and G assay procedure

ELISA plates were coated with 100 µl per well of antibody against IgA or IgG (described in section 2.2.6.2) at 1/100 in double distilled water and left at RT (20 - 25°C) for 1 hr. The plate was then washed 5 times with ELISA wash buffer to remove any unbound Ig. The plate was blocked with 200 µl of blocking solution containing 0.05 M Tris and 0.14 M NaCl to each well for 30 min at RT. Blocking prevented subsequent non-specific binding of diluted antisera and NS to the well surface. After washing a further 5 times with ELISA wash buffer, 100 µl of NS and standard bovine sera were added in duplicate to the antibody coated wells.

A preliminary optimisation study were carried out where NS were titrated from 1/10 to 1/10 000, a dilution at 1/2000 being identified as appropriate for the detection of IgA or IgG in NS. The standard sera were used at a dilution of 1/100 according to the manufacturer's suggestion. Plates were washed with 200 µl of ELISA wash buffer and 100 µl of sheep anti-bovine IgA-HRP or IgG-HRP conjugate (Bethyl labs, Cambridge, UK) was added (1/100 000) to each well before incubation for 1 hr at RT. After washing 5 times, 100 µl of TMB (3,3',5,5'-Tetramethylbenzidine) substrate solution was added to each well and the plate was incubated in the dark at RT for 15 min. The reaction was stopped by adding 2.5 M H_2SO_4 and measurement of O.D. were read at 450 nm.

2.2.7 Immunoglobulin assay validation

2.2.7.1 Precision

Reproducibility of the ELISA was demonstrated by intra-assay and inter-assay coefficient of variance (CV %). For intra-assay precision, the CV was determined as the mean of CV of duplicate results. Inter-assay precision was determined where CVs were calculated from the mean and standard variation (SD) of the NS control samples over 10 times during the use of the assay.

2.2.7.2 Accuracy

Linearity experiments are used to assess the compatibility of NS sample to be used for assaying Ig concentration. In the absence of purified sample of Ig the accuracy was determined by linearity of dilution of 3 NS samples with varying Ig concentration.

2.2.7.3 Limit of detection

The limit of detection was determined as the lowest amount of Ig that could be distinguished from the zero standards. This was determined as the Ig concentration at 3 SD away from the mean of the ELISA optical density (mean ± 3 x SD) of the zero standard.

2.2.8 One-dimensional gel electrophoresis

Ten randomly selected NS collected and processed as described in section 2.2.2 were used to analyse the nasal proteome by 1-DE on a Criterion™ electrophoresis system (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.2.8.1 Gel and buffers

Eighteen well Criterion TGX™ Precast Gels 4-15% (Bio-Rad Laboratories, Hemel Hempstead, UK) were used for 1-DE. All stock solutions and buffers (Appendix 2.4) were prepared according to manufacturer's instruction manuals (Bio-Rad Laboratories, Hemel Hempstead, UK).

2.2.8.2 Sample preparation

Prior to 1-DE analysis, protein content was measured as mentioned in section 2.2.3. Nasal secretion samples were diluted to 1/20 and mixed with Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue and 5% ß-mercaptoethanol, 710 mM) at a ratio of 1:1 and heated at 95°C for 4 min.

2.2.8.3 Gel electrophoresis

Electrophoresis was run using a standard Tris-glycine running buffer on a Criterion™ electrophoresis system at 300 V for 20 min.

2.2.8.4 Gel staining and image acquisition

After electrophoresis, gels were stained using colloidal Coomassie blue dye for 1 hour according to manufacturer's instructions (Sigma-Aldrich, Dorset, UK), and initially destained in 10% (w/v) acetic acid and 25% (v/v) methanol for 1 min, subsequently the destain solution was removed and replaced with a fresh destain for a further 24 hours. Gel images were acquired using a UMAX PowerLookIII scanner (UMAX Technologies Inc., CA, USA) under the following settings: transmissive mode, true colour RGB and a resolution of 600 dpi.

2.2.9 Statistical analysis

Statistical analyses and graphs were performed using Minitab® for windows version 17 (Minitab® Software Inc., Pennsylvania, USA). Anderson-Darling normality test were used to analyse the skewness and distribution of biochemical, enzymological and immunological results with a significance level (α) set at 0.05.

2.3 Results

2.3.1 Bovine nasal secretion

By means of using this technique of collecting NS, 5 to 12 ml of NS was absorbed and extracted by centrifugation per tampon from each cow within a sampling period of 10 min.

2.3.2 Biochemical results

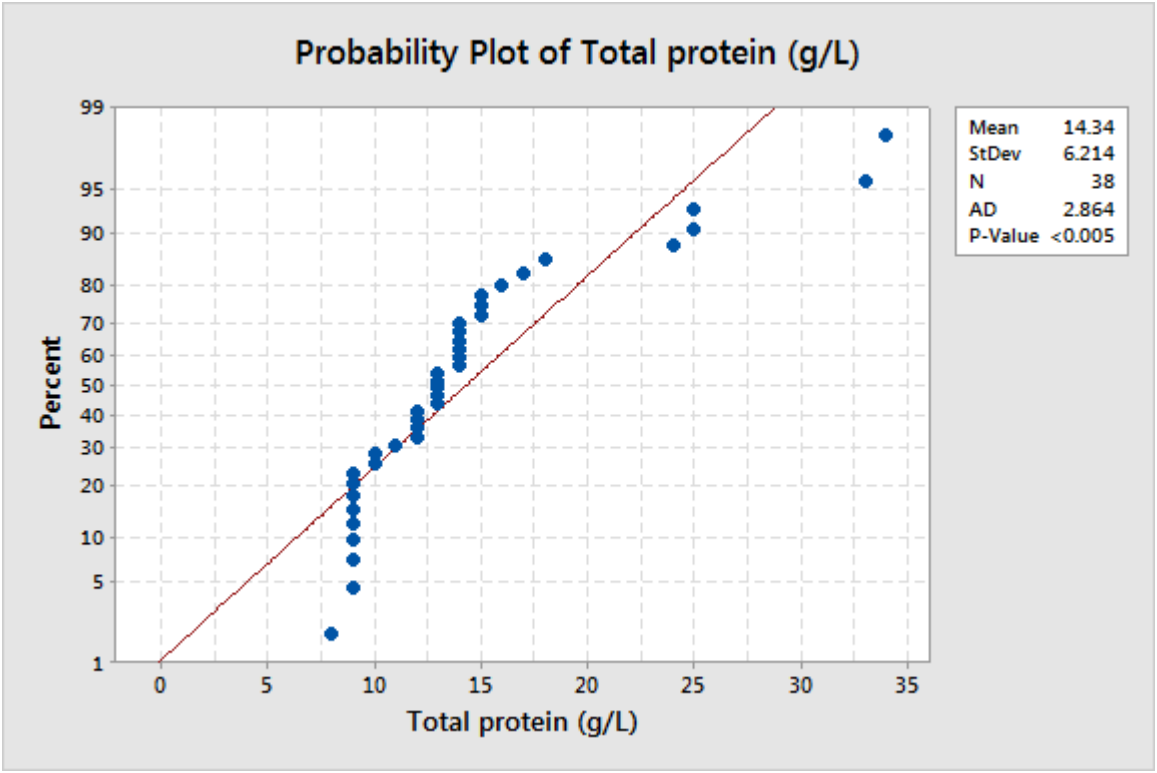
Following biochemical analysis, concentrations of total protein, creatinine, albumin and all other electrolytes were lower than the serum reference range whereas urea and total bilirubin levels were comparable with serum reference ranges (Table 2.1). Anderson-Darling statistical test for and histogram for all substrate and electrolyte measurements were given in Figure 2.3 – 2.12. Sodium, calcium and phosphate were normally distributed whereas other analytes such as total protein, albumin, potassium, chloride, urea, creatinine and total bilirubin were not normally distributed.

Substrate & Electrolyte (unit)	Median (Range)	Reference Range (Bovine serum)[a]
Urea (mmol/L)	3.1 (1.8 – 6.1)	0 - 8
Calcium (mmol/L)	1.1 (0.6 - 1.4)	2.2 – 3.3
Phosphate (mmol/L)	0.8 (0.4 - 1.6)	1.1 – 2.8
Creatinine (µmol/L)	30 (5 - 61)	53 - 132
Total bilirubin (µmol/L)	0 (0 - 2)	0 - 8
Total protein (g/L)	14 (9 - 34)	52 - 84
Albumin (g/L)	3 (0 - 5)	21 - 34
Sodium (mmol/L)	60 (18 - 98)	135 - 151
Potassium (mmol/L)	2.4 (0.6 – 3.9)	3.2 - 5.8
Chloride (mmol/L)	18.7 (14.2 - 33.7)	96 - 111

[a]Laboratory reference range for bovine serum

Table 2.1: Biochemical properties of NS from 38 apparently healthy cattle.
The values for median and range are presented.

A)



B)

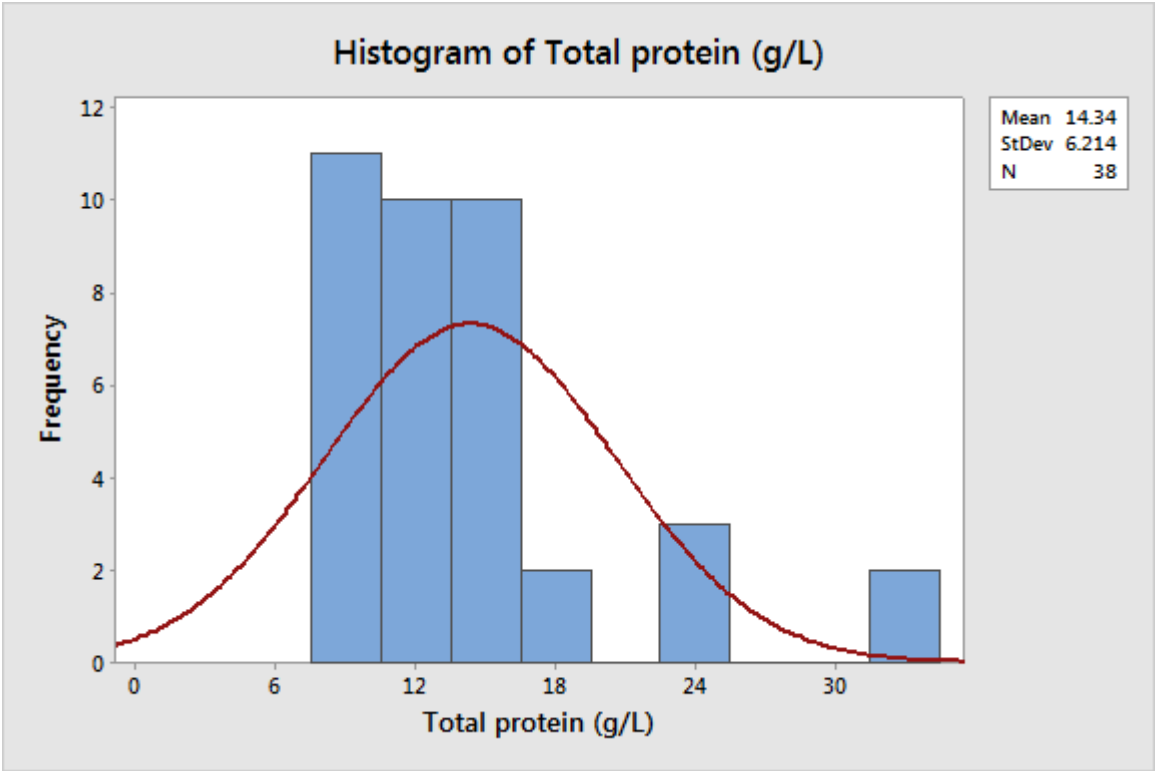
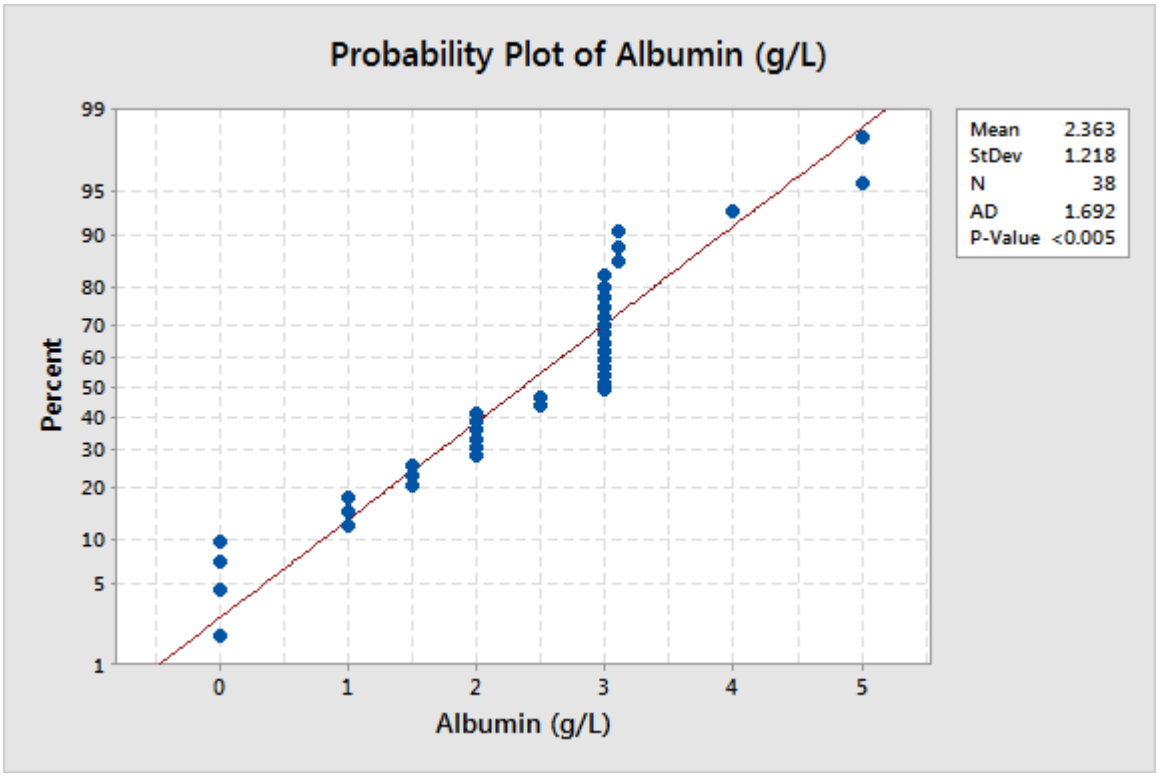


Figure 2.3: Graphs showing distribution of total protein concentration in NS from apparently healthy cattle (n=38). Total protein concentration was not normally distributed. A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of total protein concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

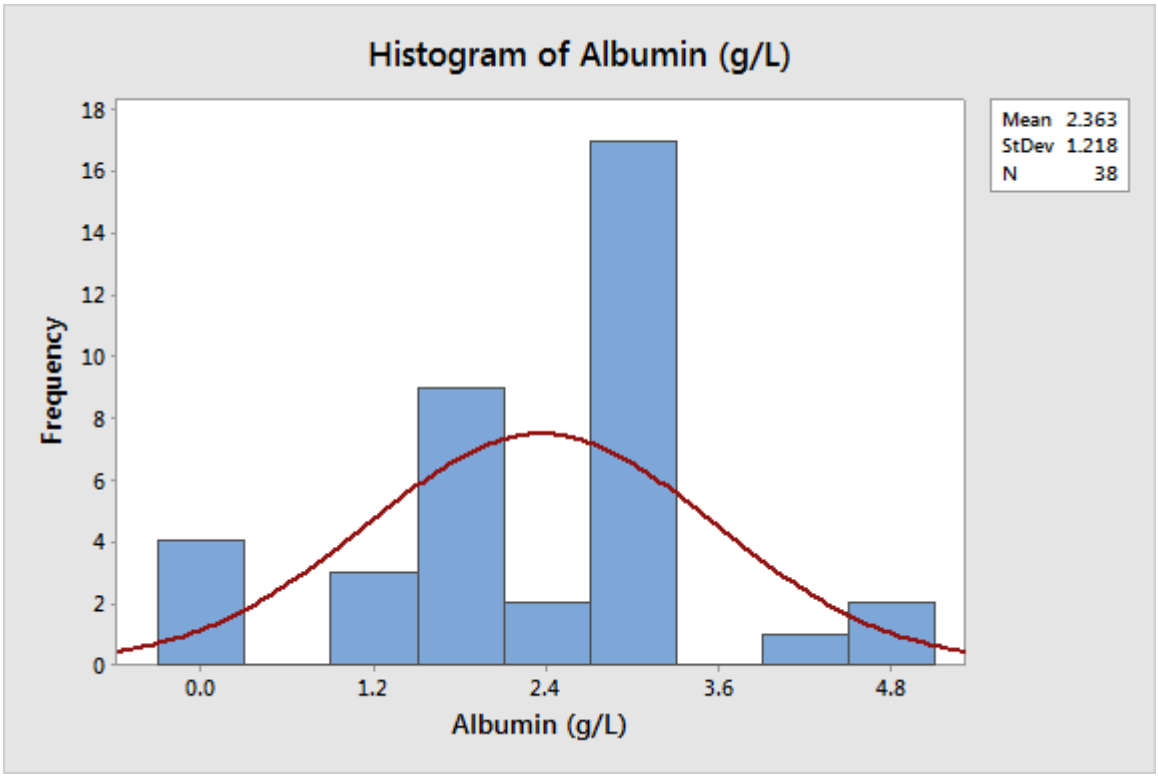
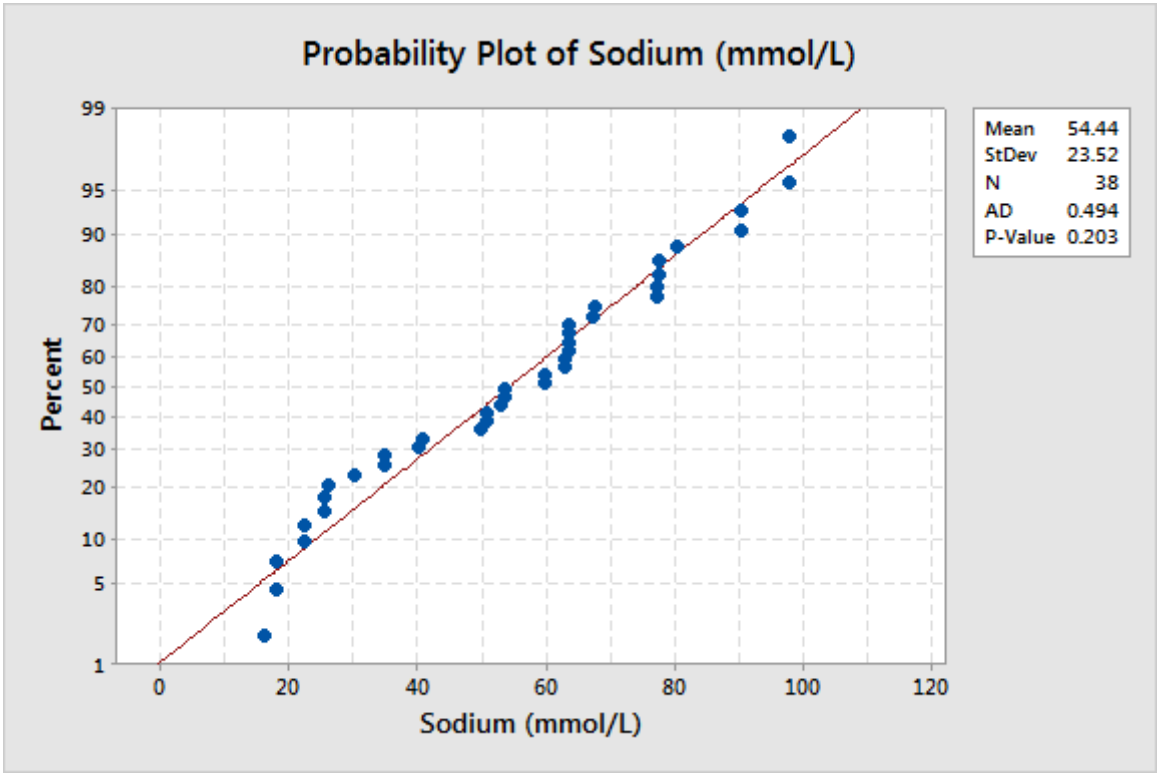


Figure 2.4: Graphs showing distribution of albumin concentration in NS from apparently healthy cattle (n=38). Albumin concentration was not normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of albumin concentration for normality check. The mean, SD, AD and p-value are presented.

A)



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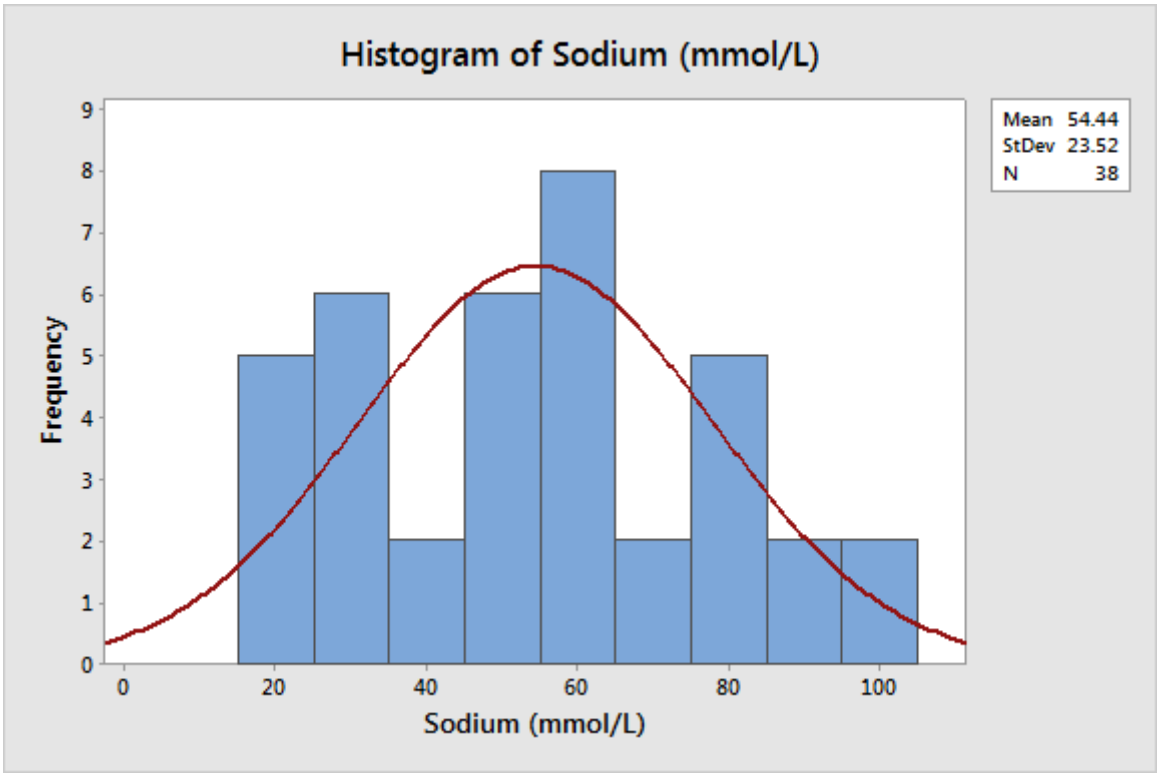
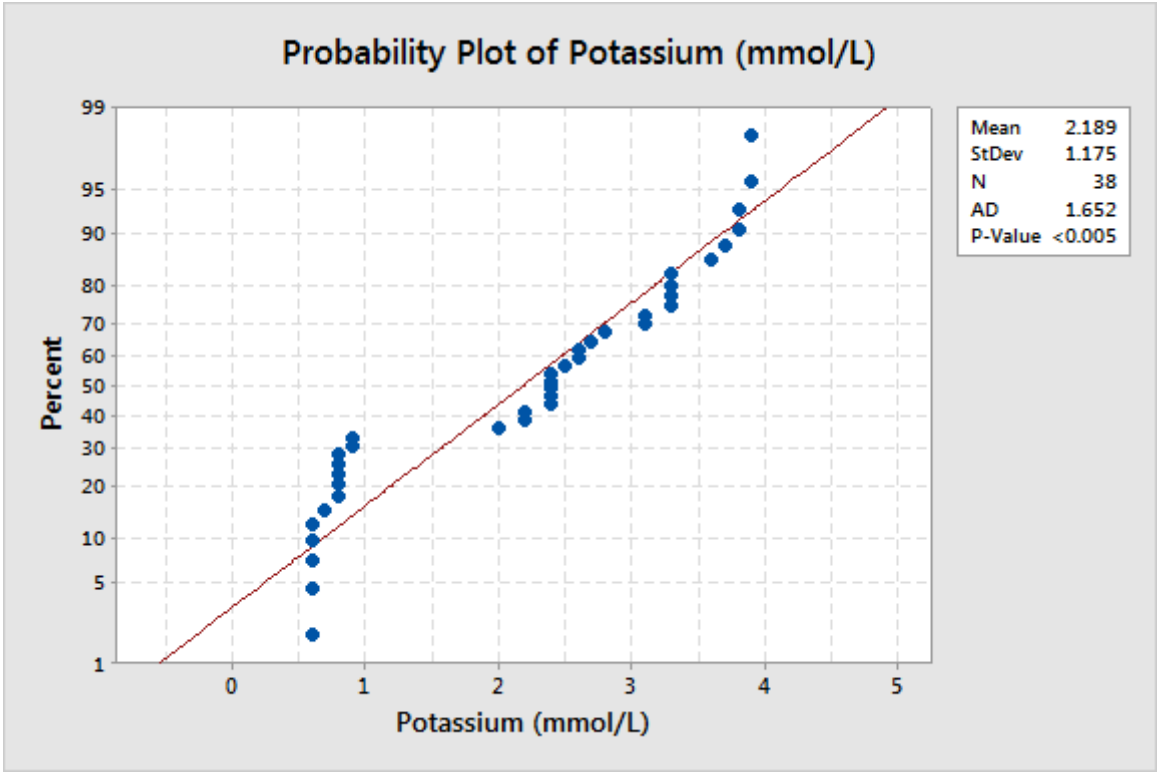


Figure 2.5: Graphs showing distribution of sodium concentration in NS from apparently healthy cattle (n=38). Sodium concentration was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of sodium concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

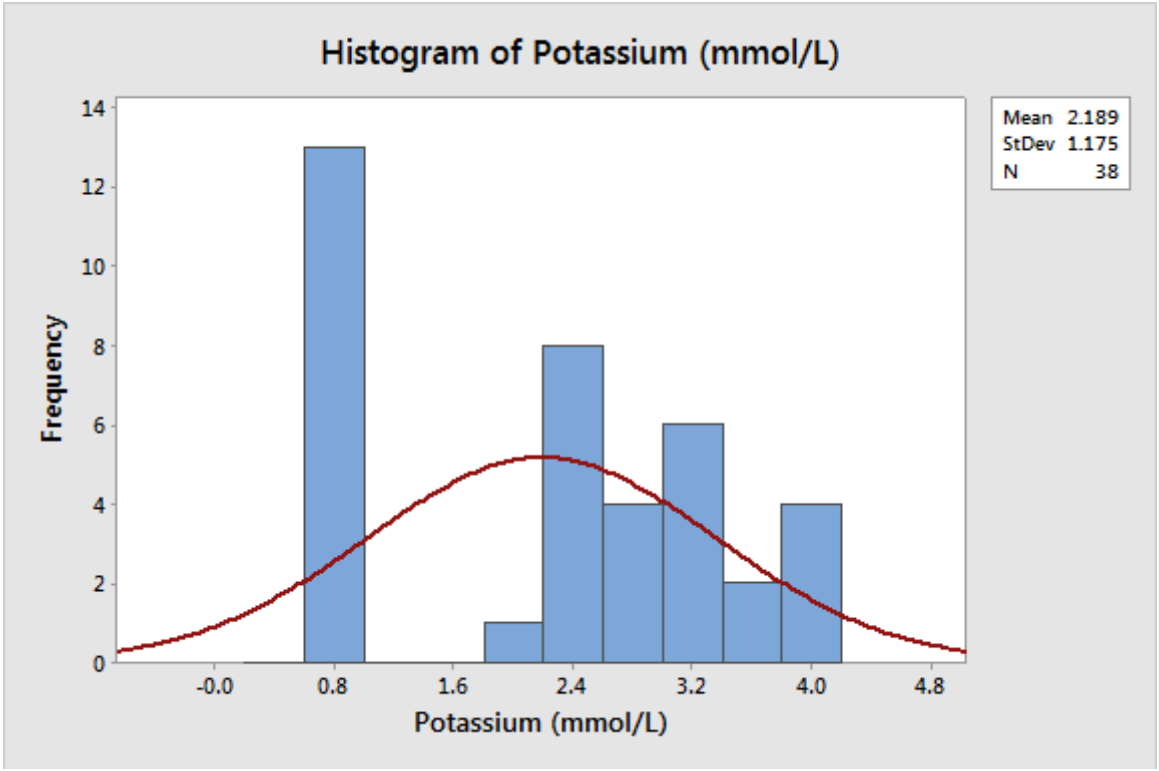
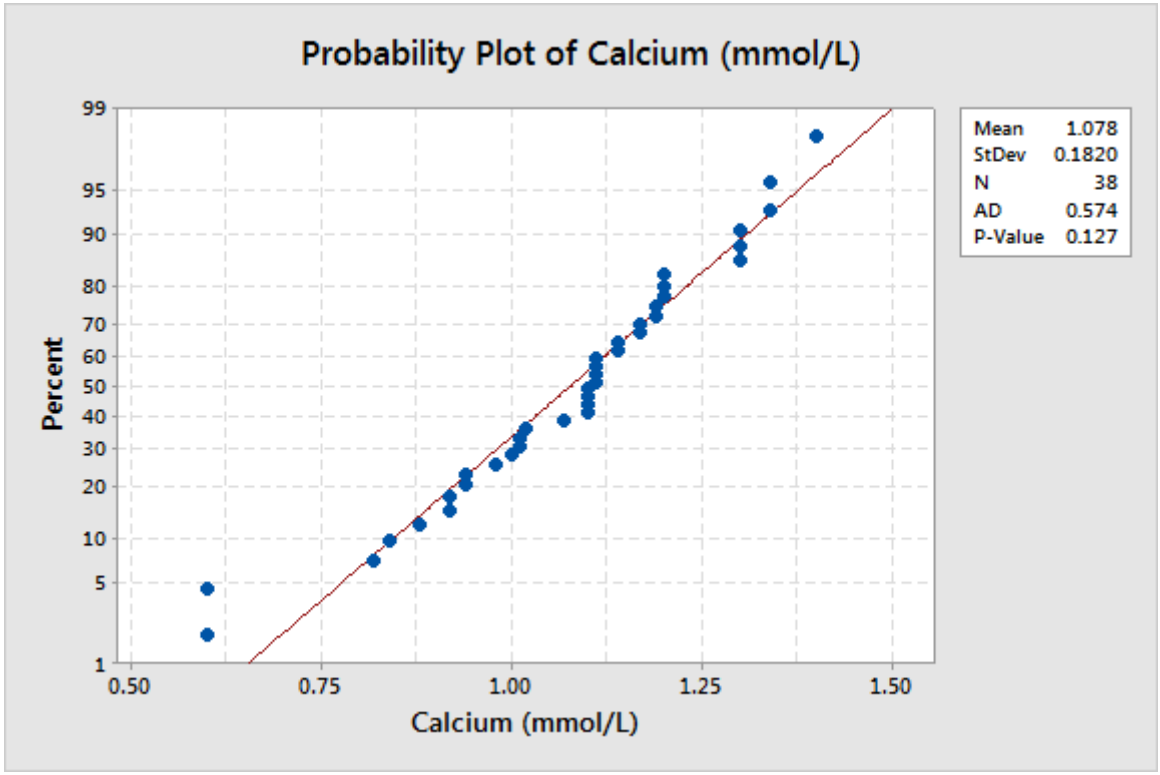


Figure 2.6: Graphs showing distribution of potassium concentration in NS from apparently healthy cattle (n=38). Potassium concentration was not normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of potassium concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

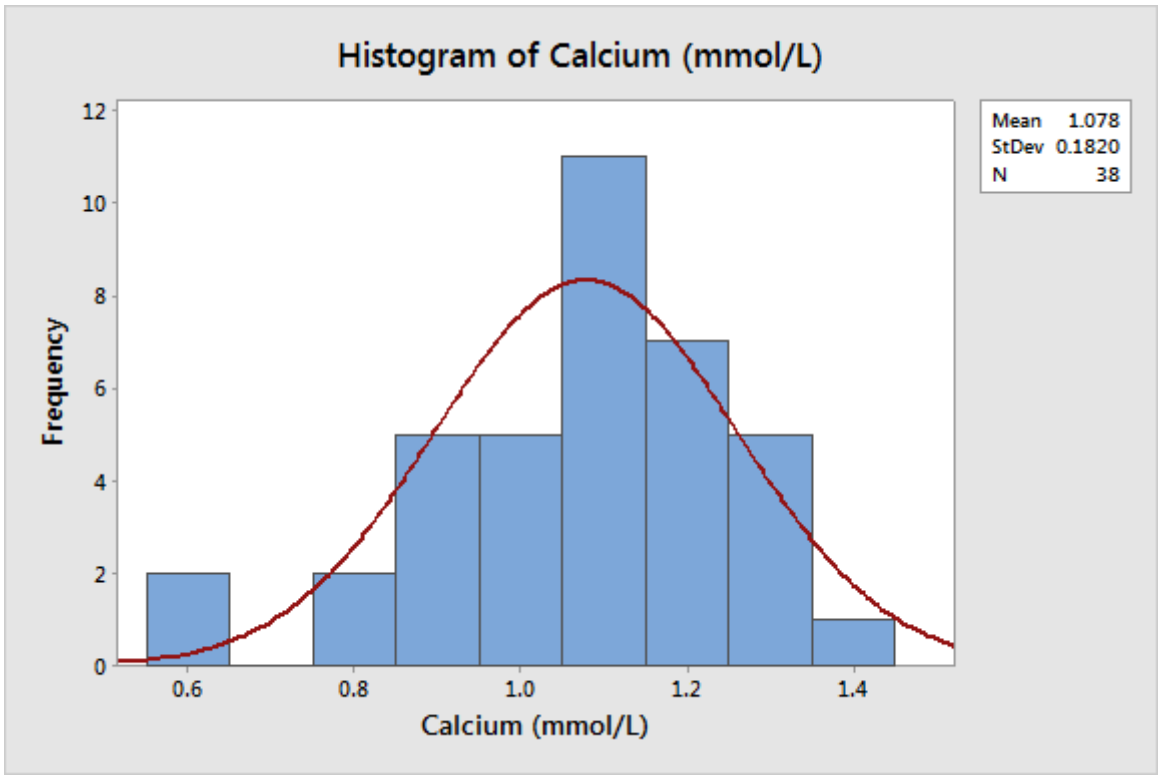
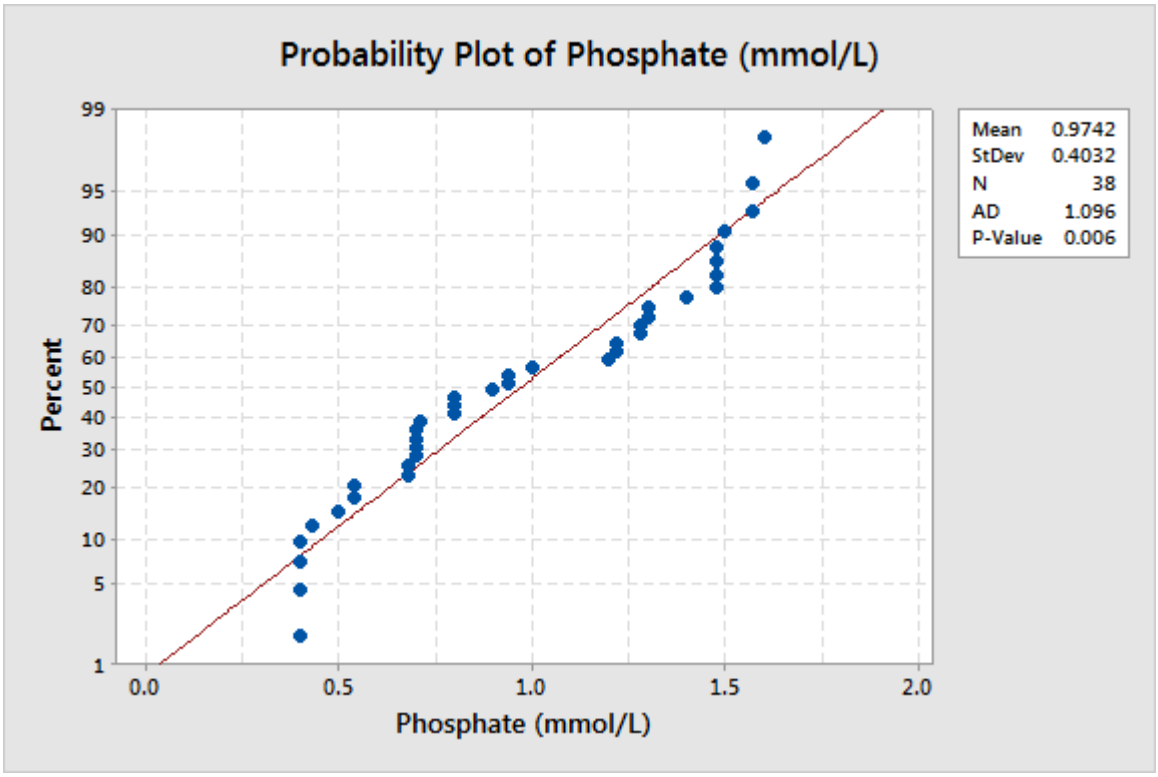


Figure 2.7: Graphs showing distribution of calcium concentration in NS from apparently healthy cattle (n=38). Calcium concentration was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of calcium concentration for normality check. The mean, SD, AD and p-value are presented.

A)



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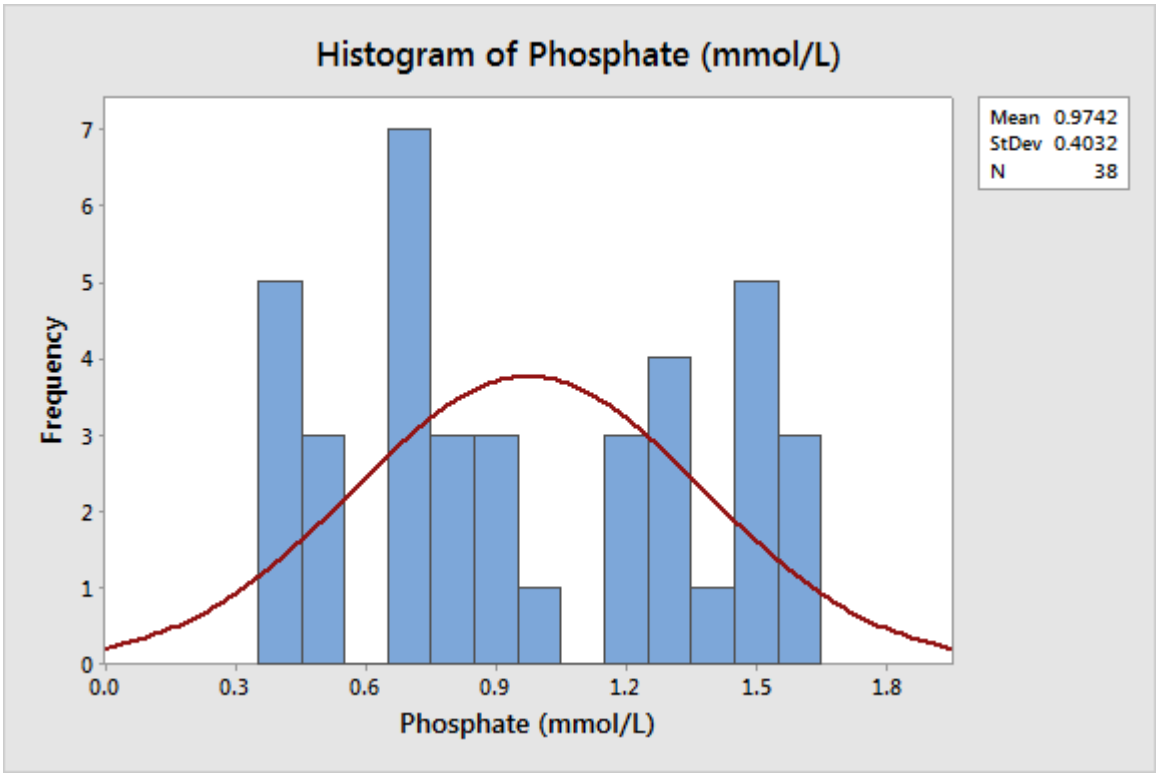
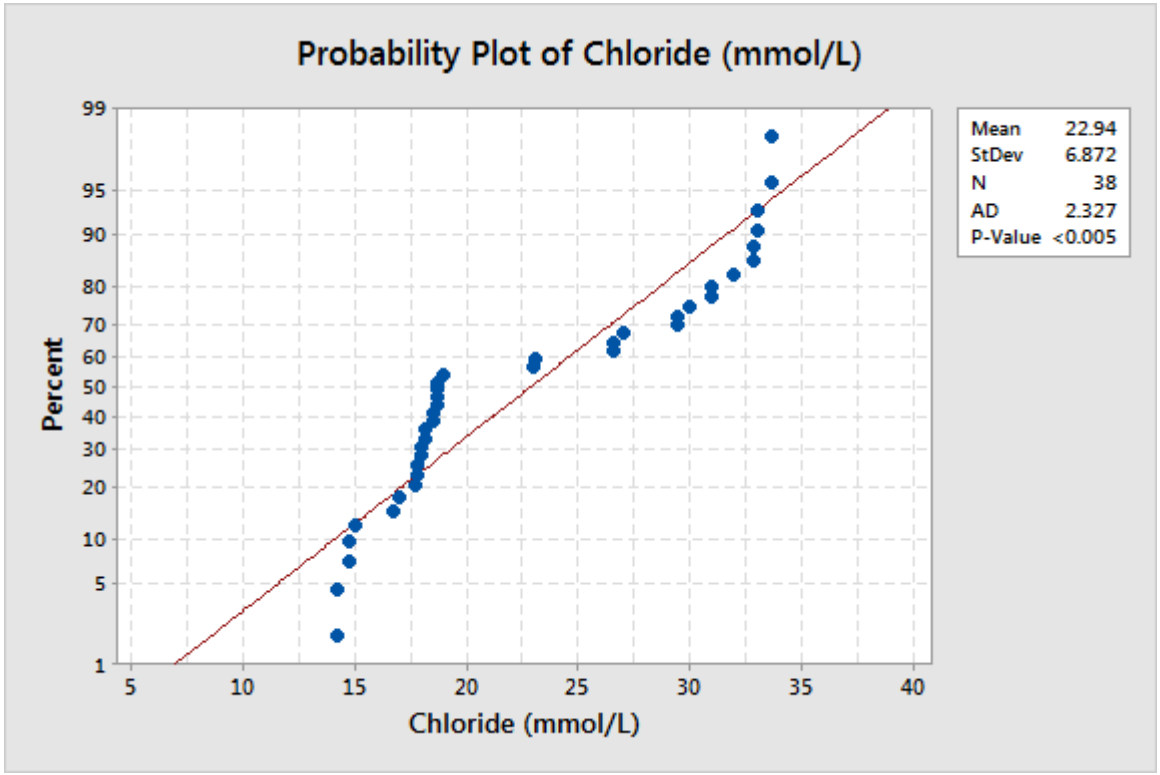


Figure 2.8: Graphs showing distribution of phosphate concentration in NS from apparently healthy cattle (n=38). Phosphate concentration was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of phosphate concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

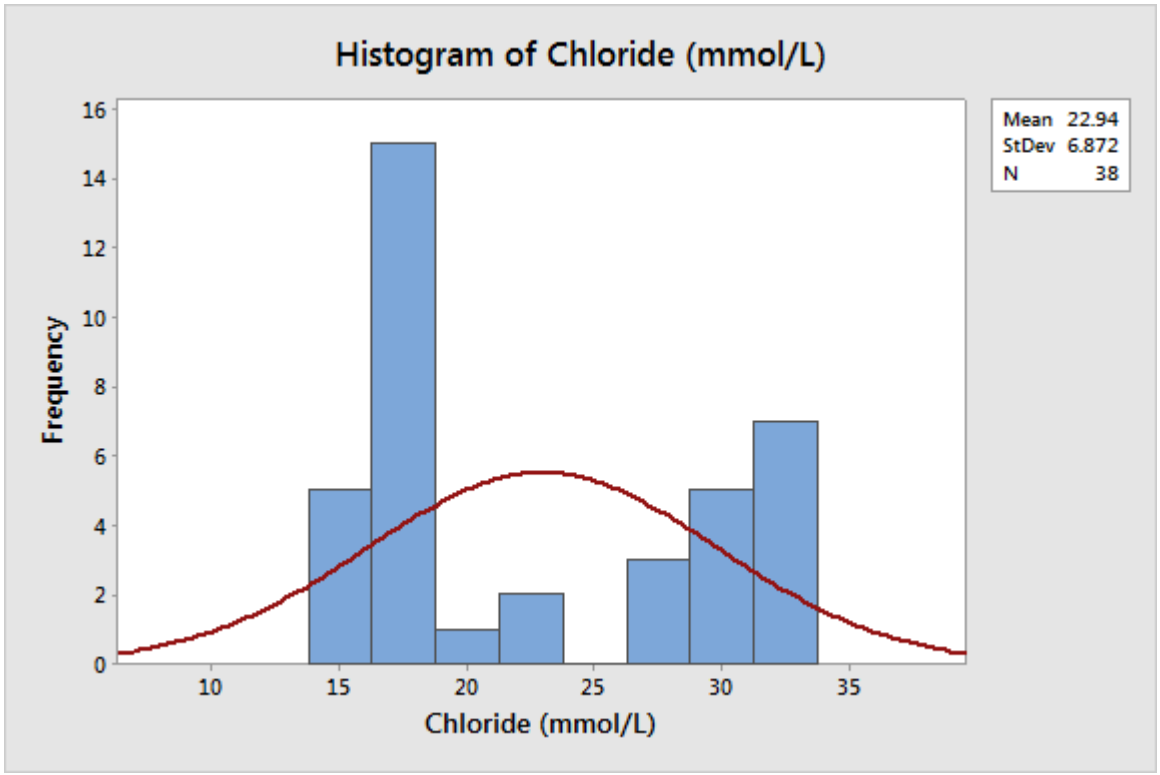
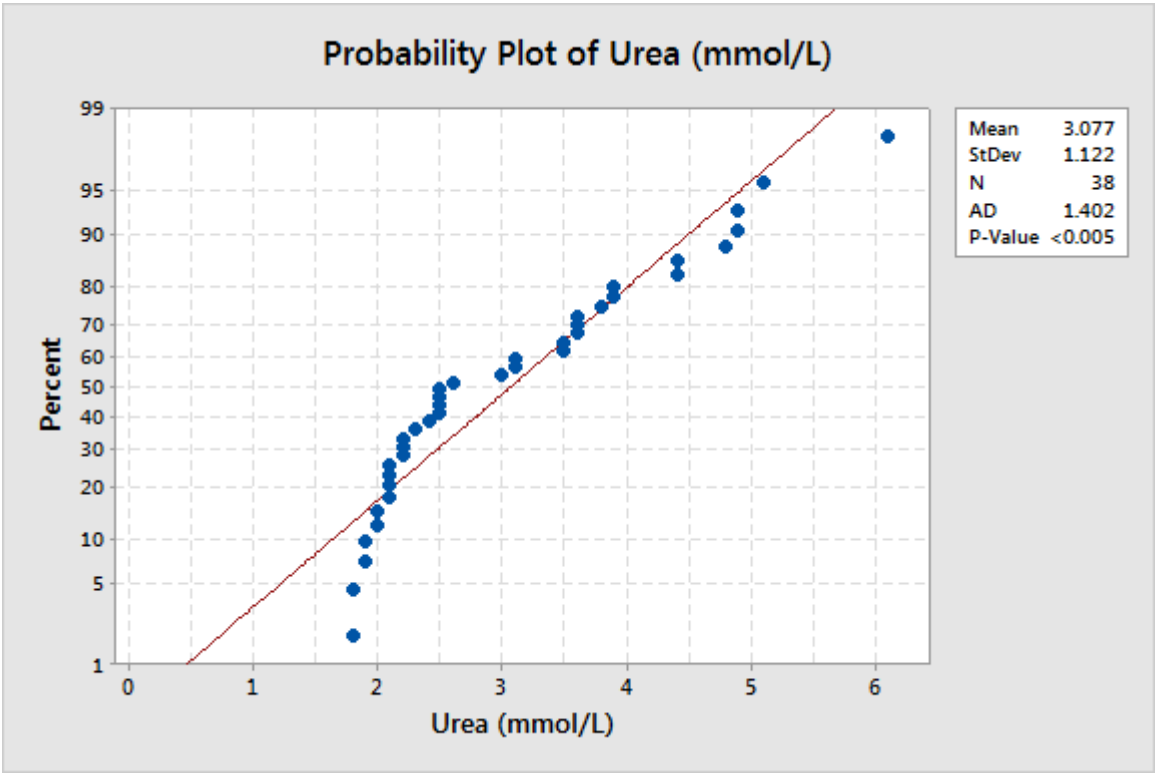


Figure 2.9: Graphs showing distribution of chloride concentration in NS from apparently healthy cattle (n=38). Chloride concentration was not normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of chloride concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

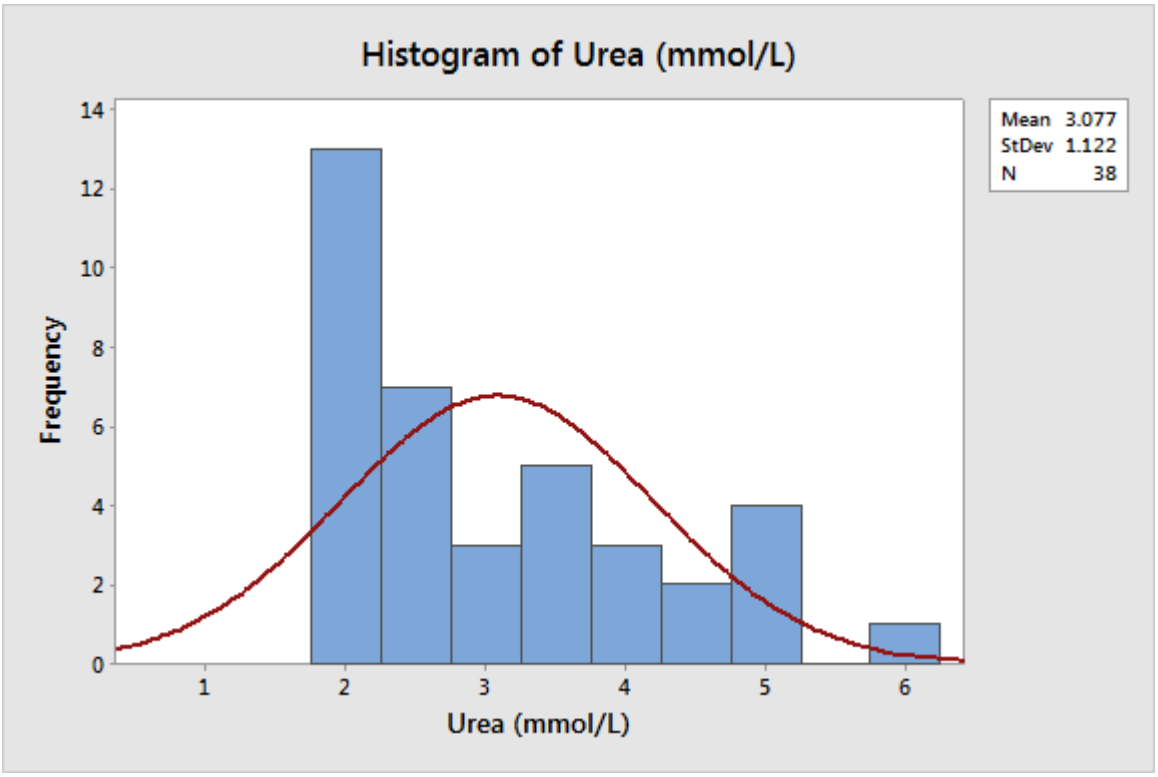
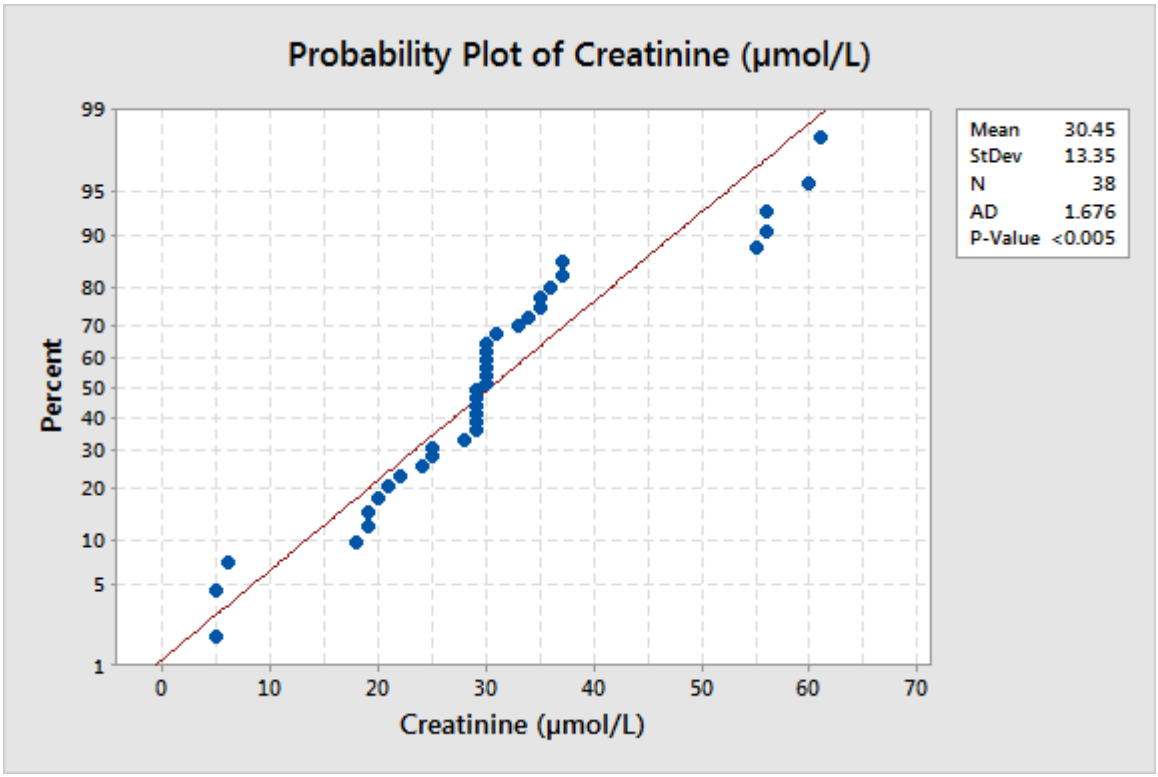


Figure 2.10: Graphs showing distribution of urea concentration in NS from apparently healthy cattle (n=38). Urea concentration was not normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of urea concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

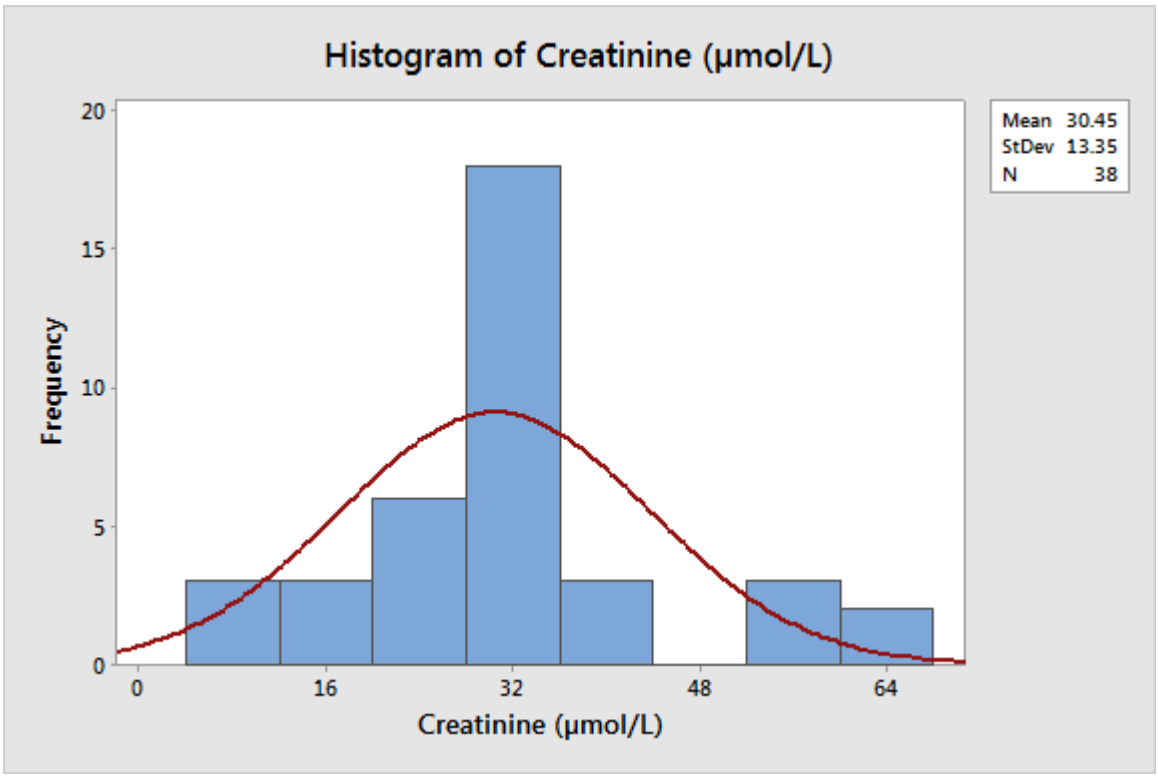
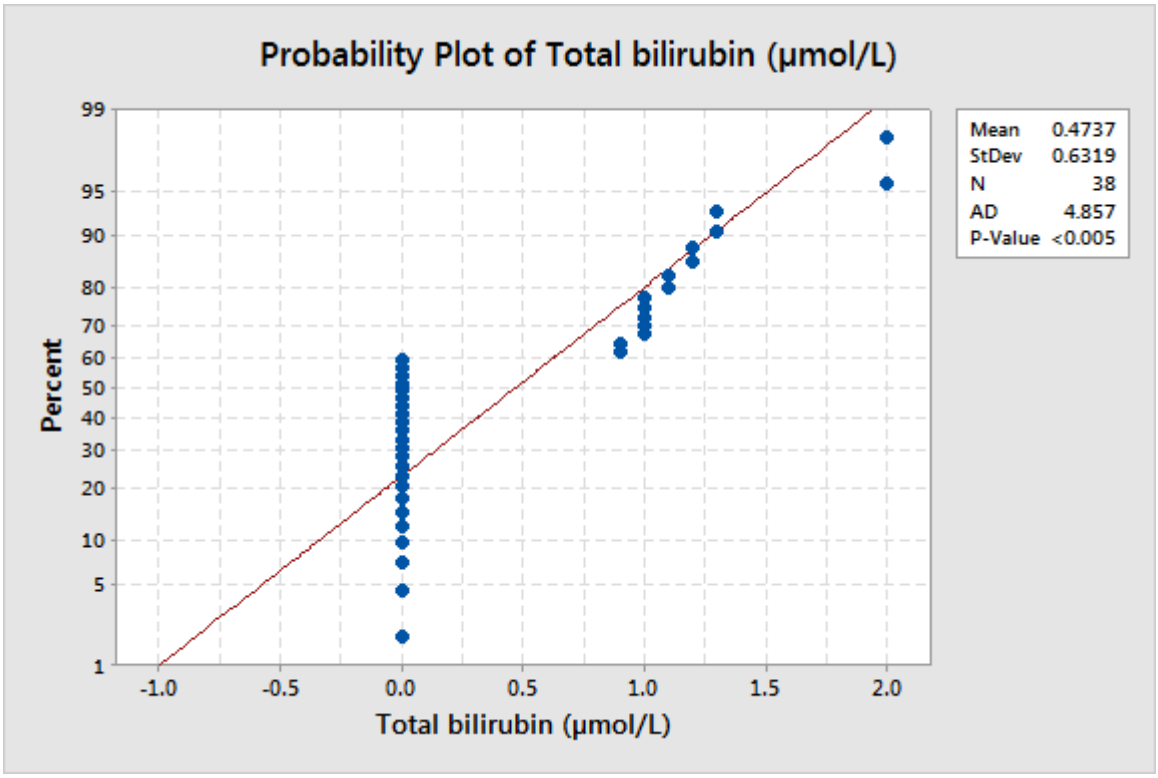


Figure 2.11: Graphs showing distribution of creatinine concentration in NS from apparently healthy cattle (n=38). Creatinine concentration was not normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of creatinine concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

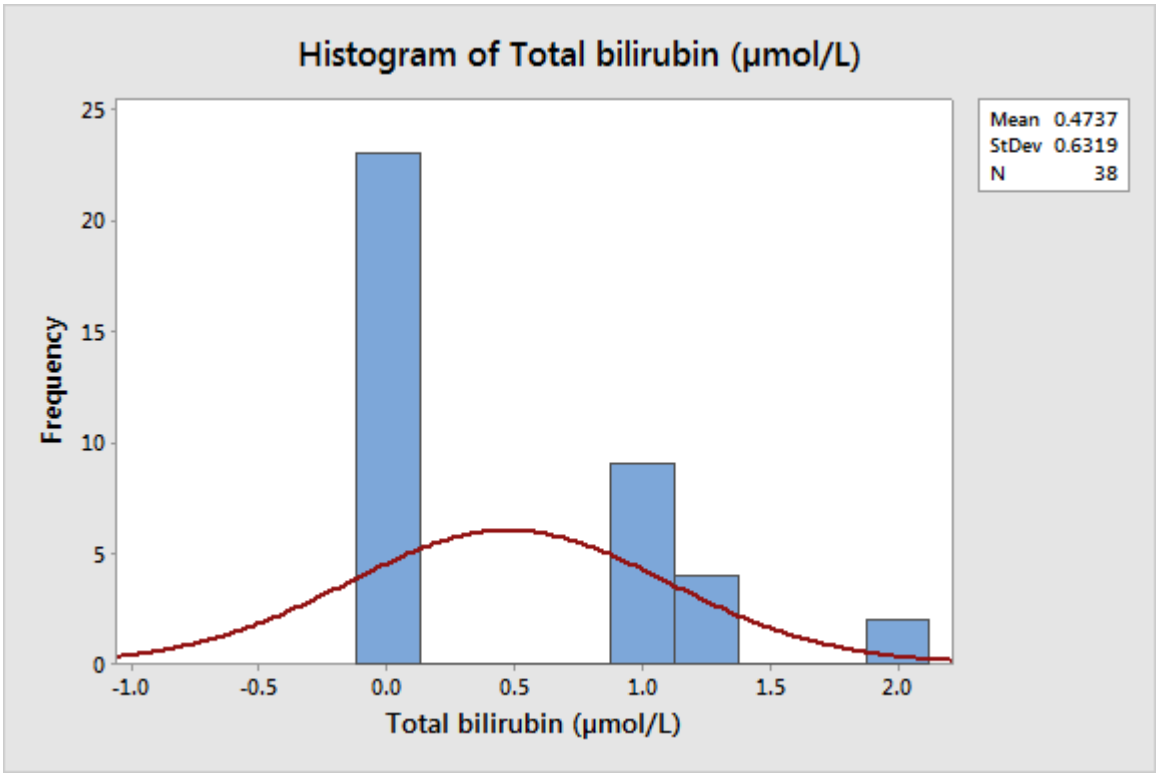


Figure 2.12: Graphs showing distribution of total bilirubin concentration in NS from apparently healthy cattle (n=38). Total bilirubin concentration was not normally distributed.

A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of total bilirubin concentration for normality check. The mean, SD, AD and p-value are presented.

2.3.3 Enzymological results

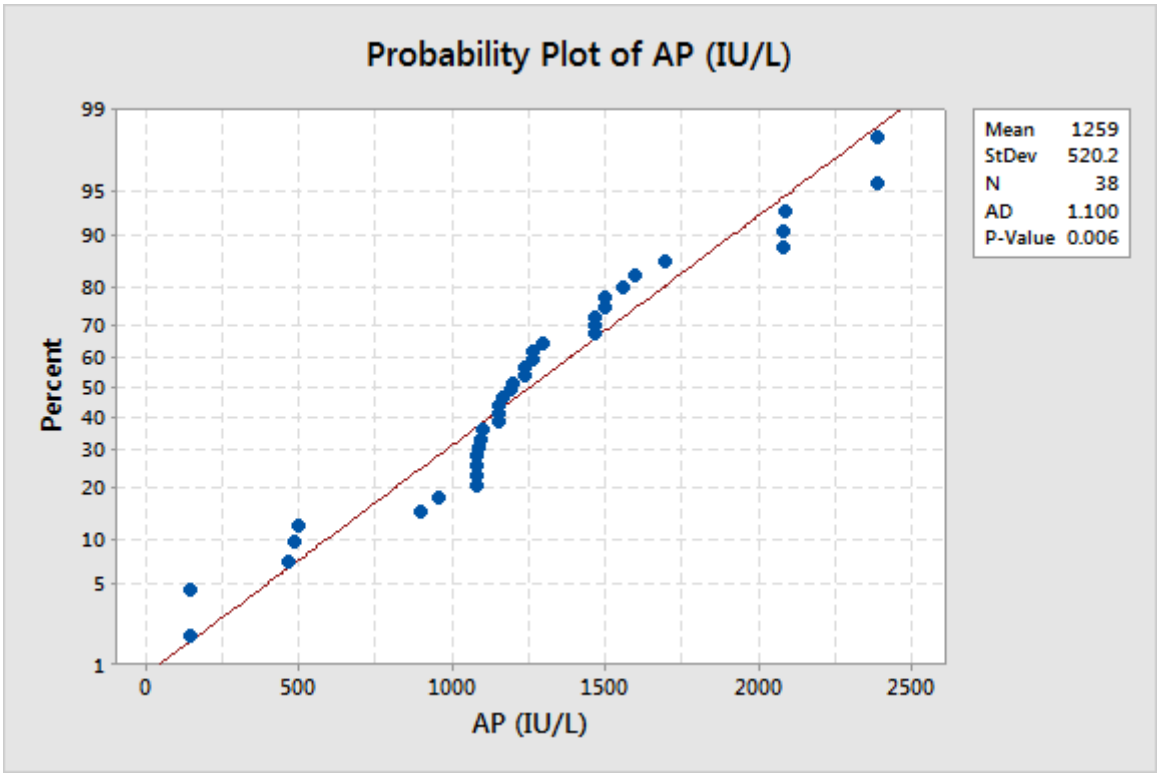
The mean AP activity in NS was 15.7-fold higher at 80:1259 than the serum reference range for AP (Table 2.2) while GGT activity was 2.5-fold higher at 27:67 than the bovine serum GGT reference range. However, the other enzyme AST levels were comparable with serum reference ranges. Anderson-Darling statistical test and histogram for all enzymes were given in Figure 2.13 – 2.15. All enzymes in bovine NS were normally distributed.

Enzymes (unit)	Median (Range)	Reference Range (Bovine serum)[a]
AP (IU/L)	1155 (144 - 2392)	20 - 80
AST (IU/L)	97 (50 - 184)	10 - 140
GGT (IU/L)	68 (28 - 109)	0 - 27

[a]Laboratory reference range for bovine serum

Table 2.2: Enymes activity of bovine NS taken from healthy animals (n=38).
The values for median and range were presented.

A)



B)

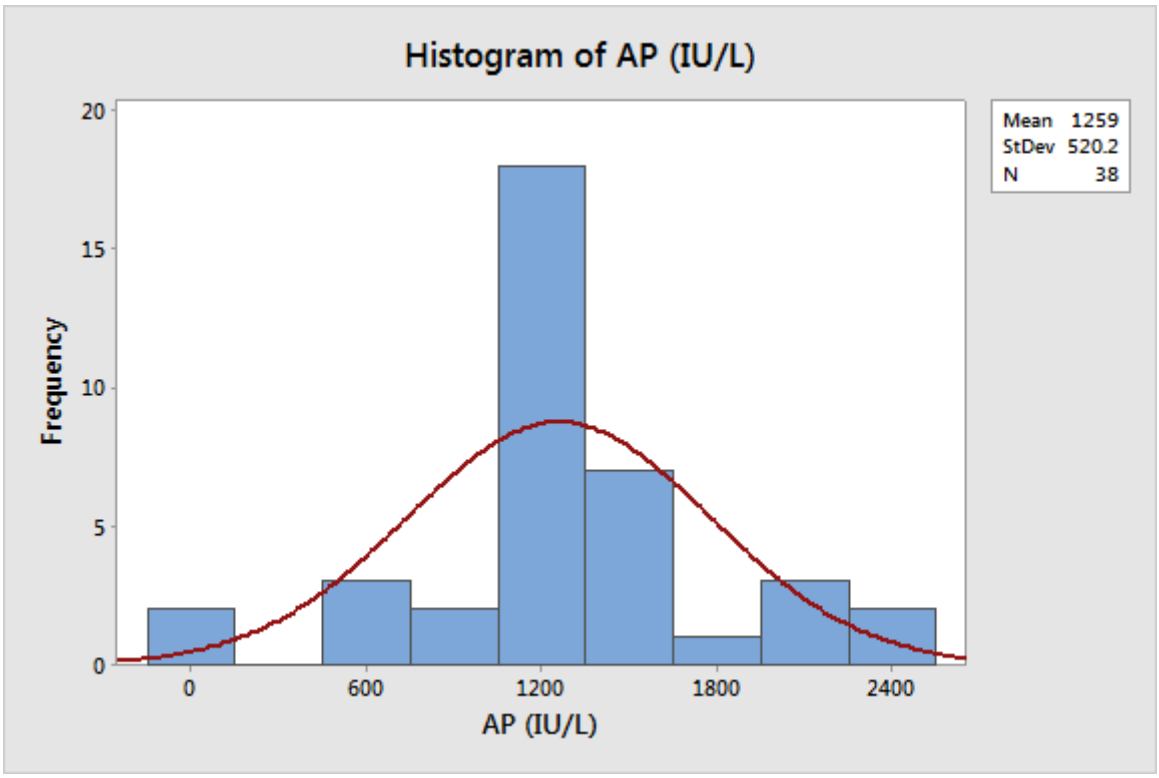
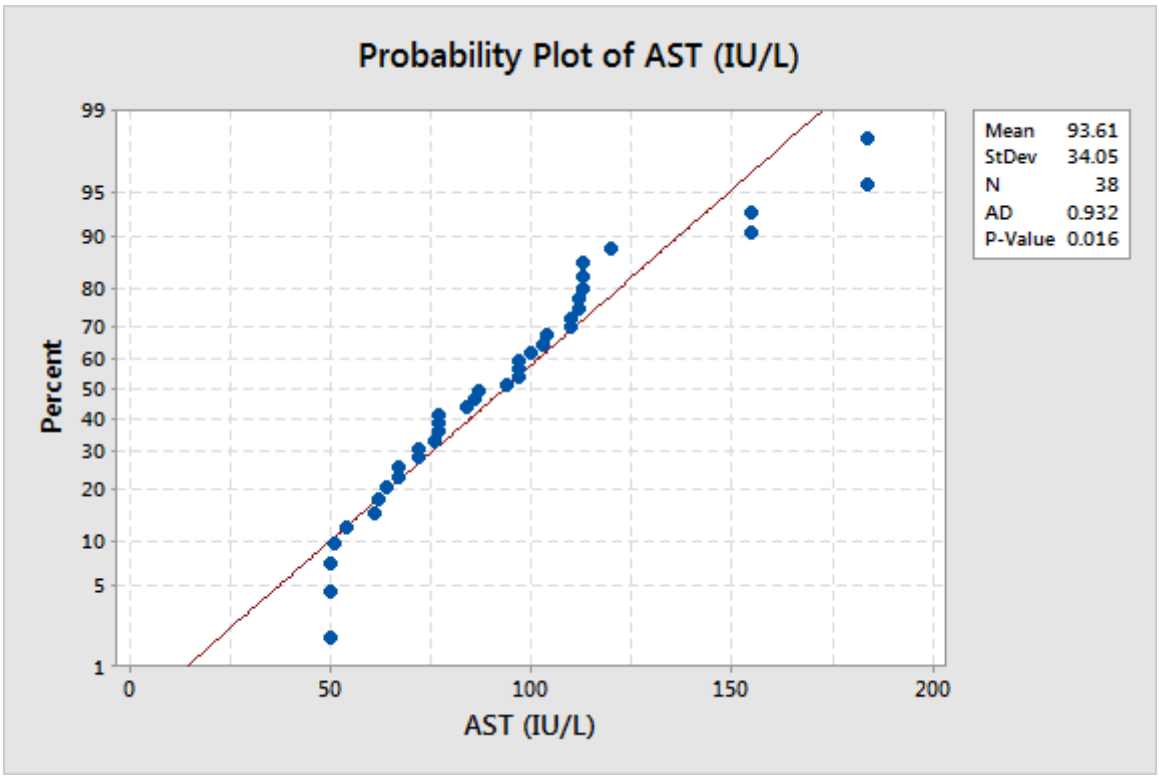


Figure 2.13: Graphs showing distribution of AP activity in NS from apparently healthy cattle (n=38). Alkaline phosphatase activity was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of AP activity for normality check. The mean, SD, AD and p-value are presented.

A)



B)

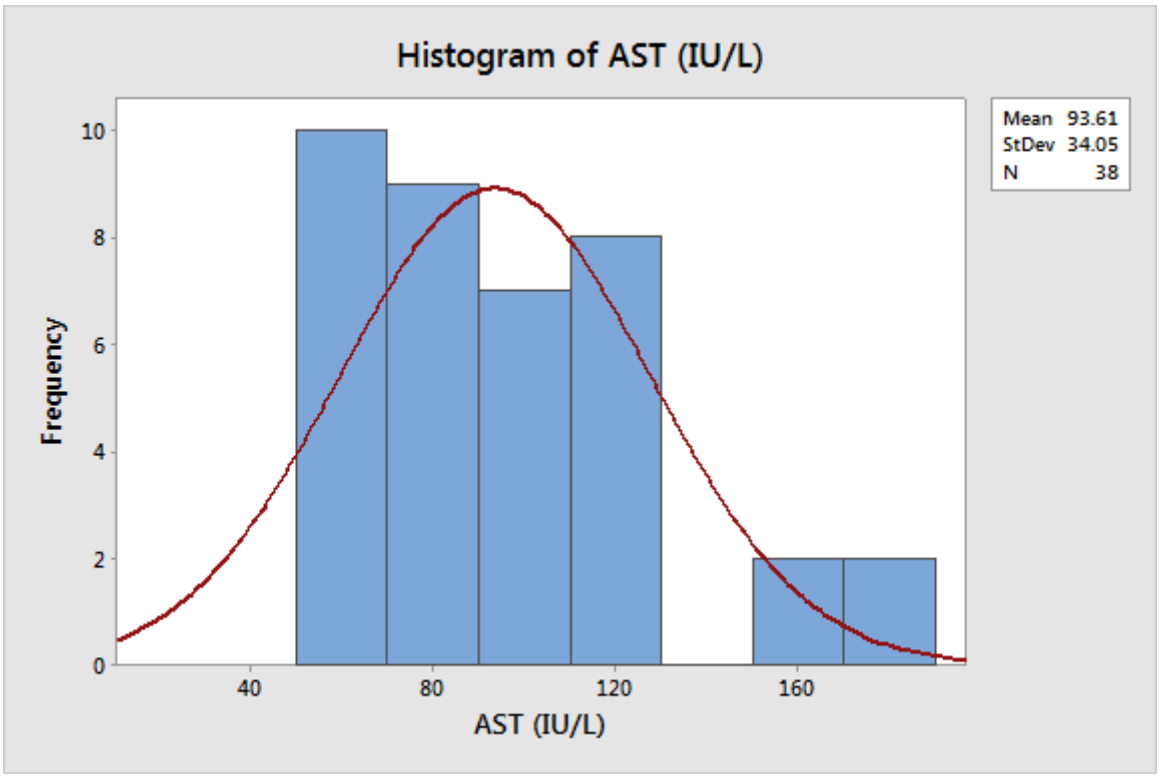
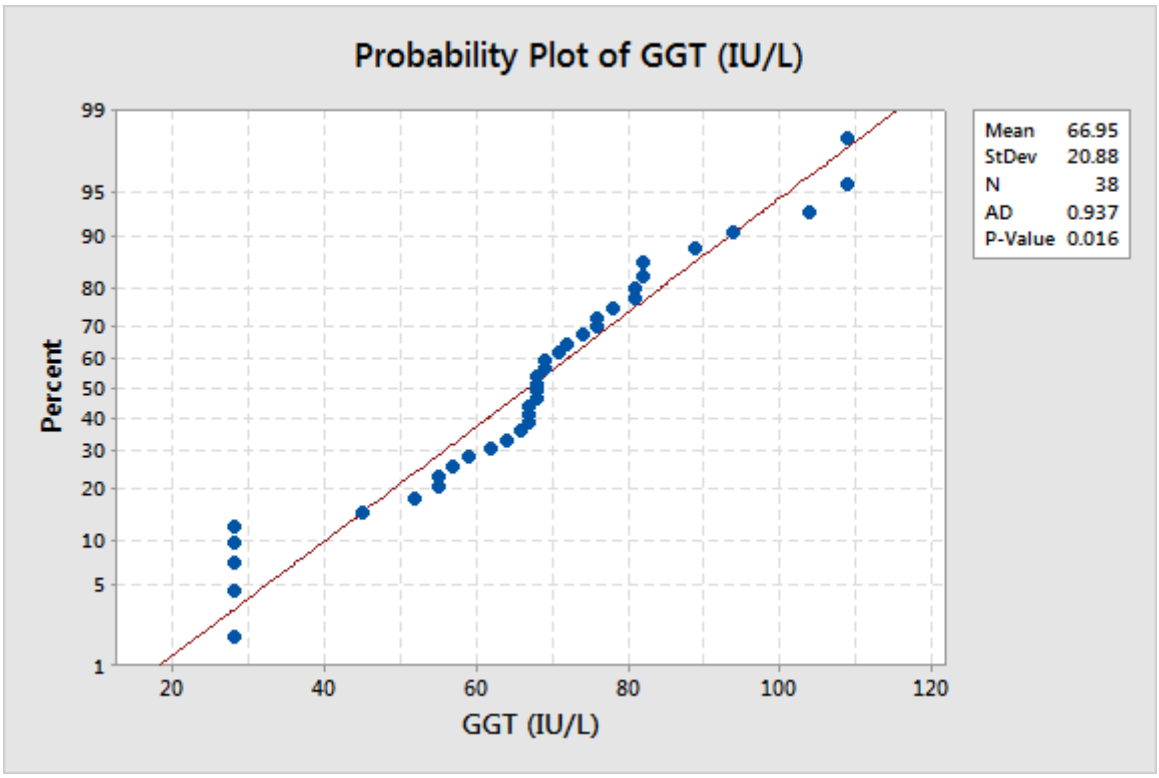


Figure 2.14: Graphs showing distribution of AST activity in NS from apparently healthy cattle (n=38). Aspartate transaminase activity was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of AST activity for normality check. The mean, SD, AD and p-value are presented.

A)



B)

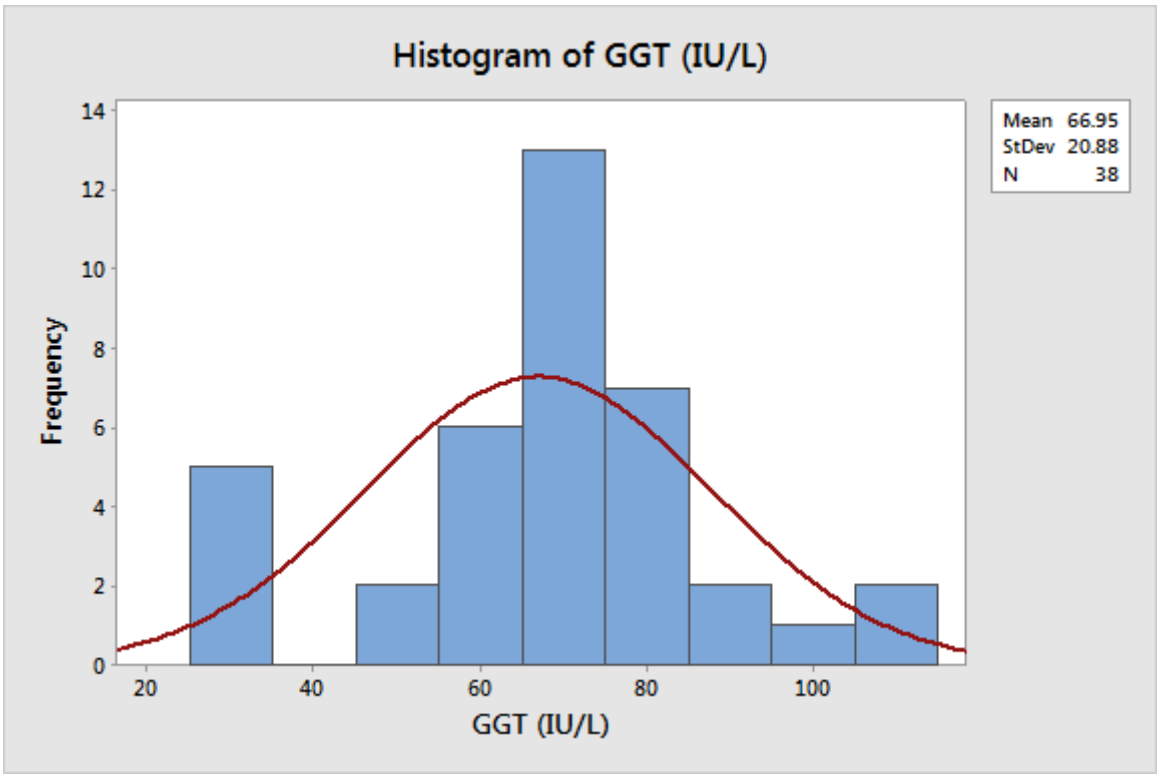


Figure 2.15: Graphs showing distribution of GGT activity in NS from apparently healthy cattle (n=38). Gamma-glutamyl transferase activity was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of GGT activity for normality check. The mean, SD, AD and p-value are presented.

2.3.4 Immunological analysis

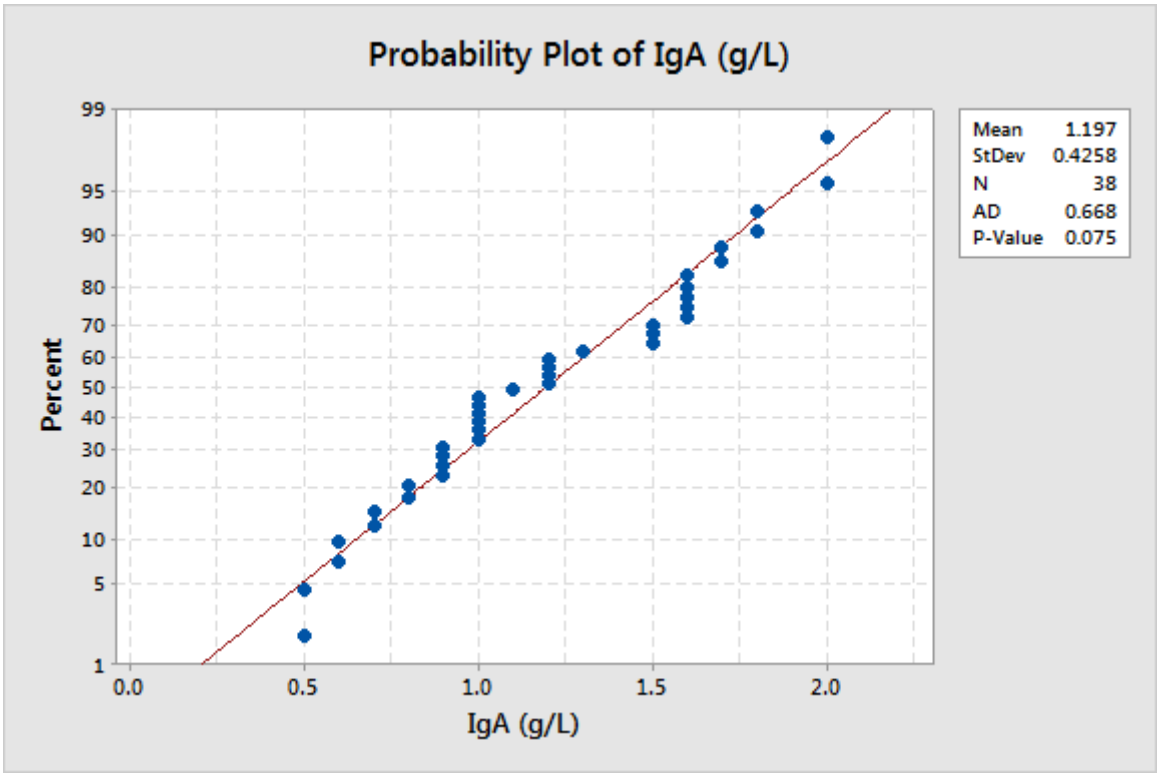
The concentrations of IgA and IgG in bovine NS were 0.5 – 2.0 g/L and 0.2 - 1.9 g/L respectively (Table 2.3). Anderson-Darling statistical test and histogram for both immunoglobulins were given in Figure 2.16 and 2.17. Immunoglobulin A concentration was normally distributed whereas IgG concentration was not normally distributed.

Immunoglobulins (unit)	Median (Range)	Reference Range
IgA (g/L)	1.2 (0.5 – 2.0)	1.8 - 3.9 [a]
IgG (g/L)	0.5 (0.2 - 1.9)	0.1 - 0.7 [b]

[a] Morgan et al., 1981; [b] Duncan *et al.*, 1972

Table 2.3: Imunoglobulin A and G concentrations in bovine NS taken from apparently healthy cattle (n=38).
The values for median and range are presented.

A)



B)

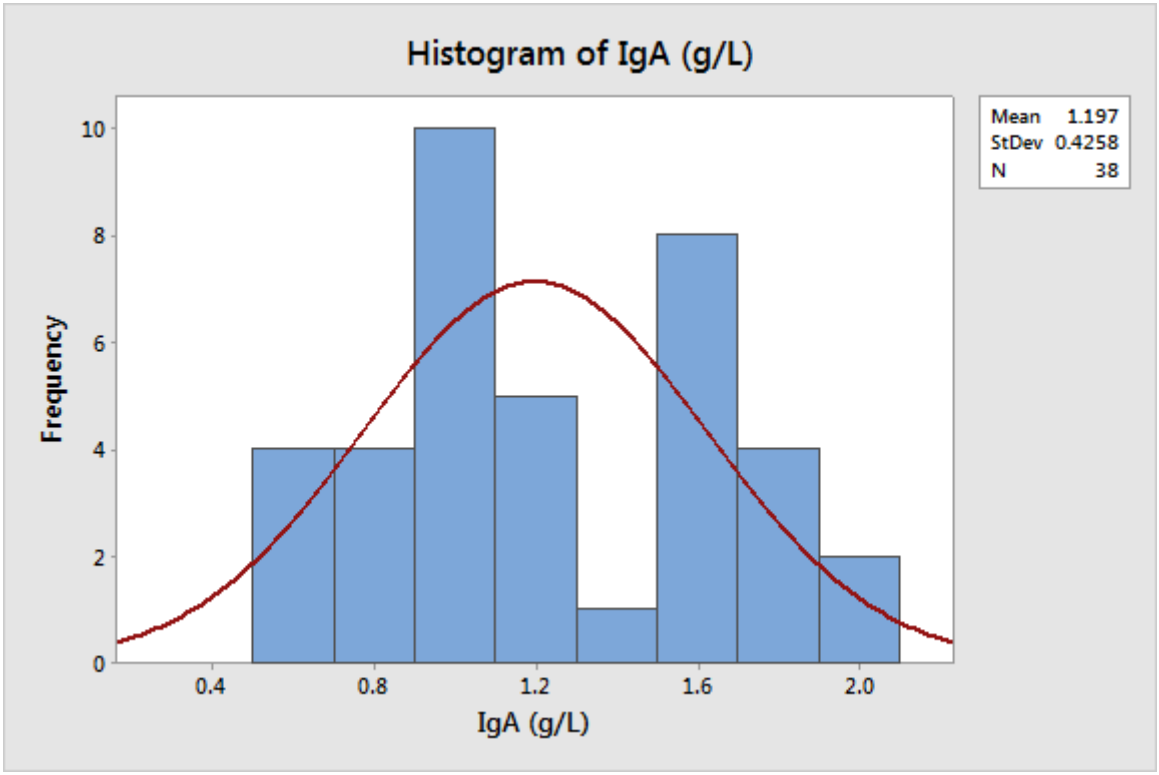
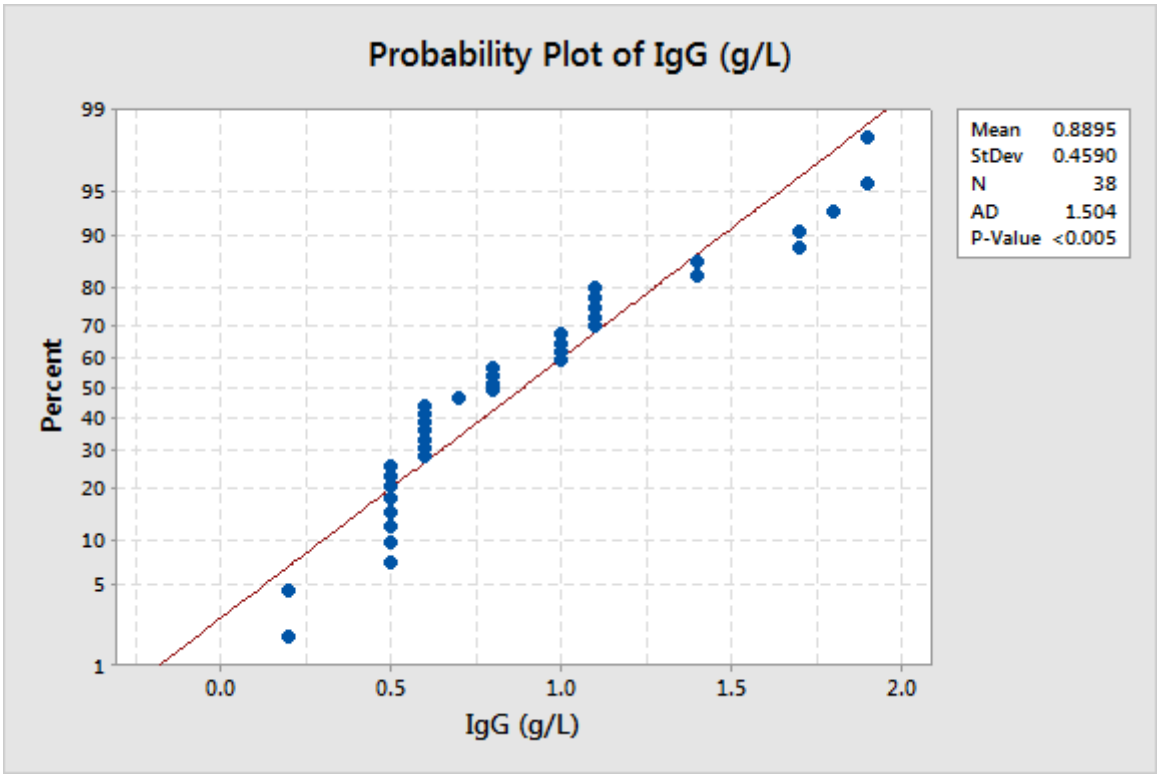


Figure 2.16: Graphs showing distribution of IgA concentration in NS from apparently healthy cattle (n=38). The IgA concentration was normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of IgA concentration for normality check. The mean, SD, AD and p-value are presented.

A)



B)

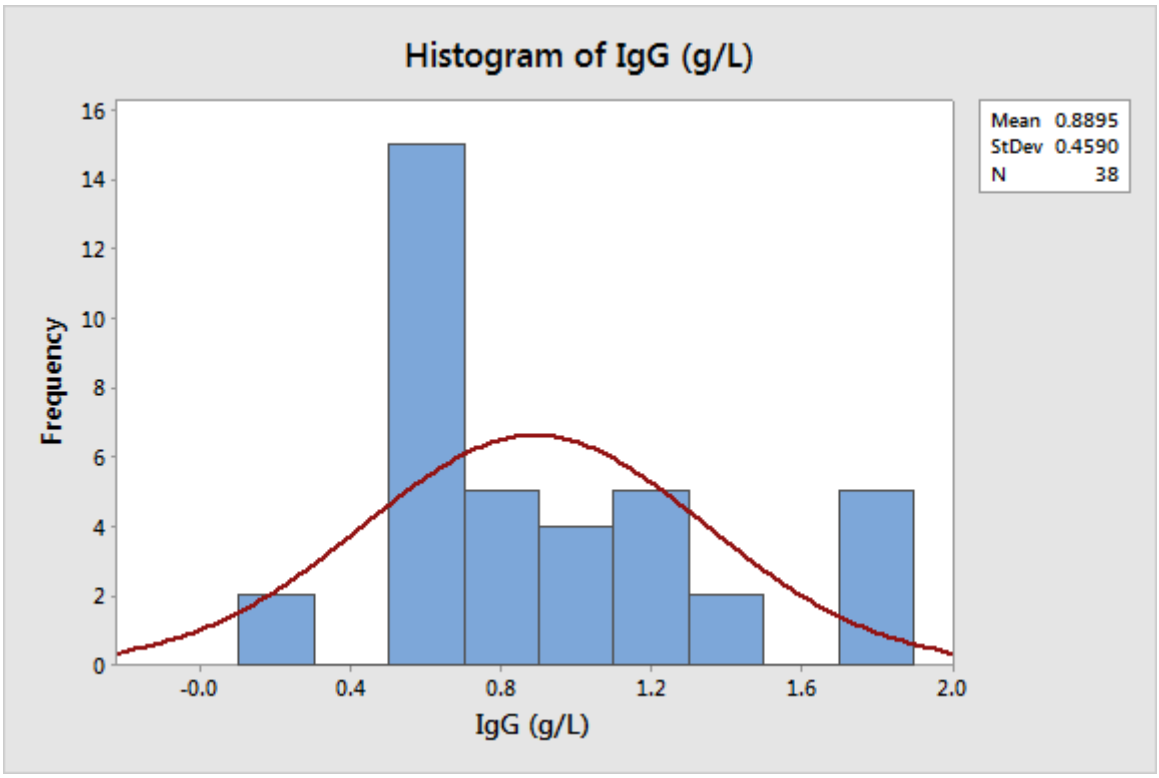


Figure 2.17: Graphs showing distribution of IgG concentration in NS from apparently healthy cattle (n=38). The IgG concentration was not normally distributed.
A) Anderson-Darling (AD) test for normality with p-value <0.05 is considered not normally distributed and B) Histogram of IgG concentration for normality check. The mean, SD, AD and p-value are presented.

2.3.5 Assay optimisation

Throughout the optimisation procedure for Ig assays, a wide range of NS dilutions were tested (Figure 2.18) however a dilution at 1/2000 was implemented for the detection of IgA or IgG in NS as this gave the steepest standard curve. The standard sera were used at a dilution of 1/100 according to the manufacturer's suggestion and the standard curves used were given at Figure 2.19. Figure 2.20 show the linearity of mean pooled NS samples (n=38) in comparison with IgA and IgG standard curve.

2.3.6 Assay validation

2.3.6.1 Intra and Inter-assay precision

The intra-assay CV for IgA and IgG assays were 10.2% and 8.5% respectively which was calculated as the mean of duplicate samples (n=38). The inter-assay values, means and the calculated CVs which were 14.2% and 23.9% for IgA and IgG respectively. The control CVs were 8.1% and 15.3% for IgA and IgG respectively.

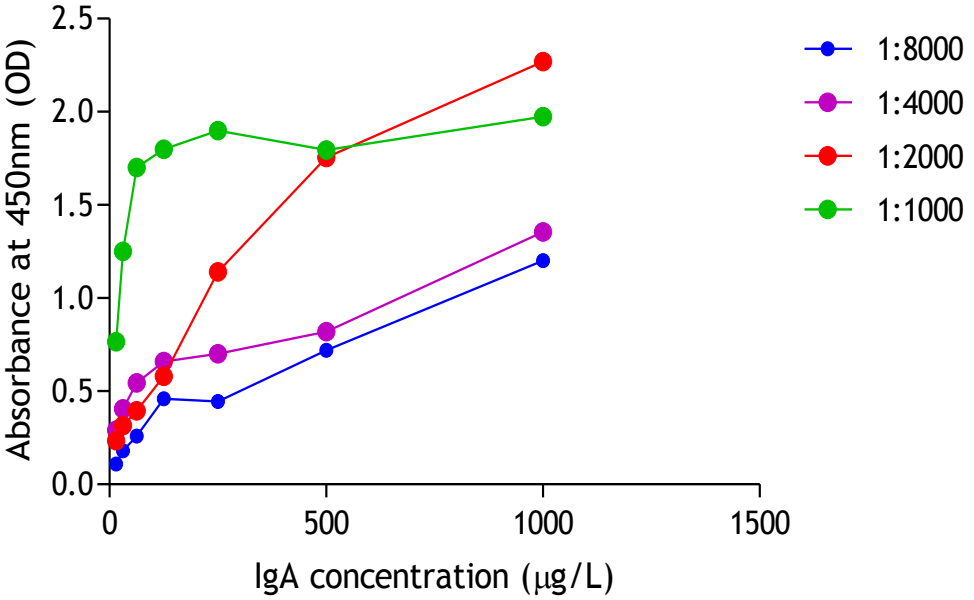
2.3.6.2 Accuracy

Serial dilution of NS samples was used to assess the assay accuracy. The samples show linearity then plateau at low dilution and are parallel to standard curve (Figure 2.20). Diluted sample concentrations were taken from the linear portion of the curve as it is the most precise and accurate area of the curve.

2.3.6.3 Limit of detection

The minimum detection limit significantly different from the blank samples was taken as the Ig concentration at 3 SD from the mean of blank sample (n=4). This resulted in a limit of detection of 0.34 and 0.45 for IgA and IgG in NS respectively.

A)



B)

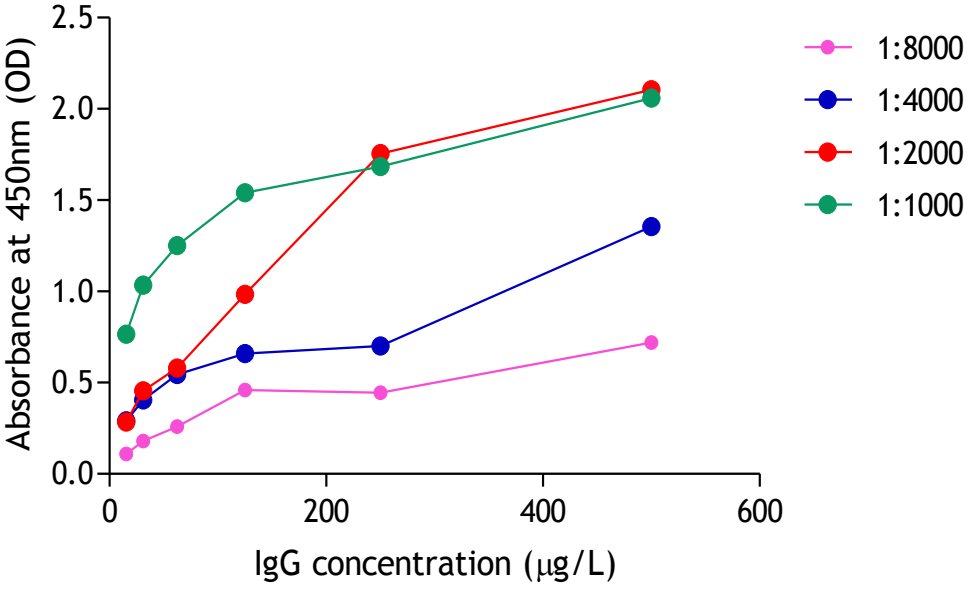
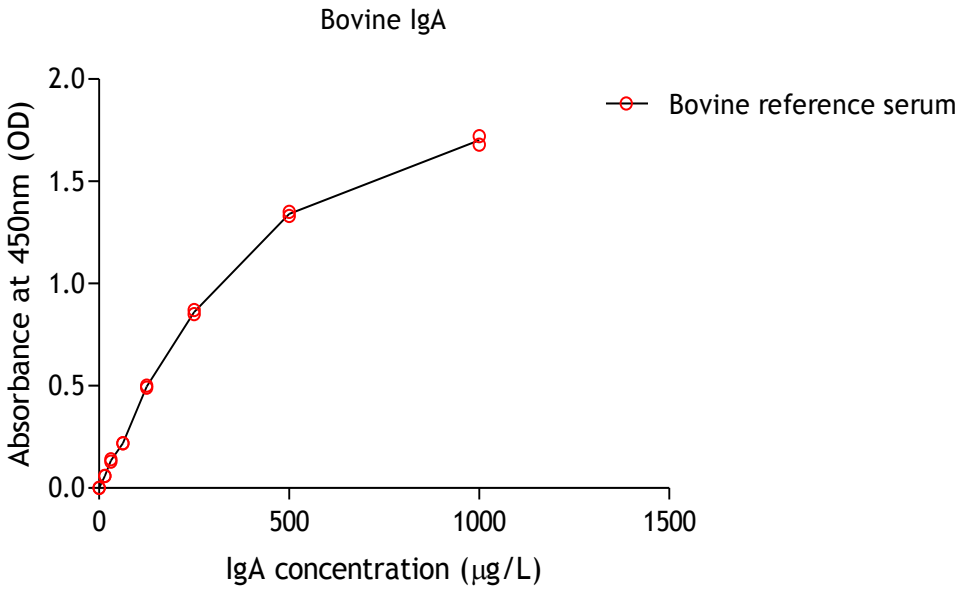


Figure 2.18: Optimisation of bovine NS concentration for ELISA.
The effect of NS dilutions at 1/1000; 1/2000; 1/4000 and 1/8000 on the A) IgA and B) IgG standard curve. Nasal secretion sample diluted at 1/2000 was chosen to analyse Ig concentration.

A)



B)

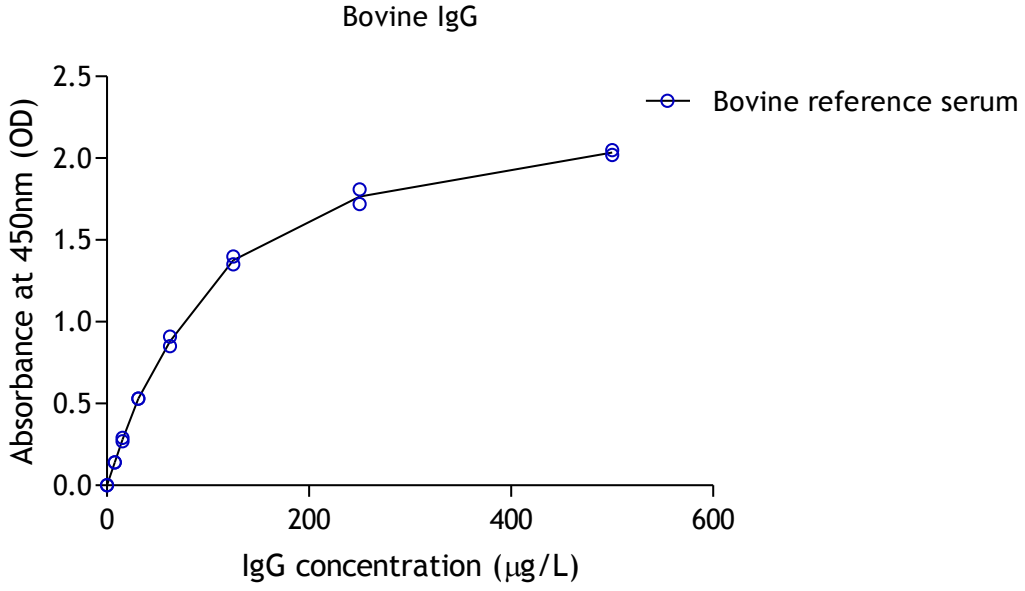
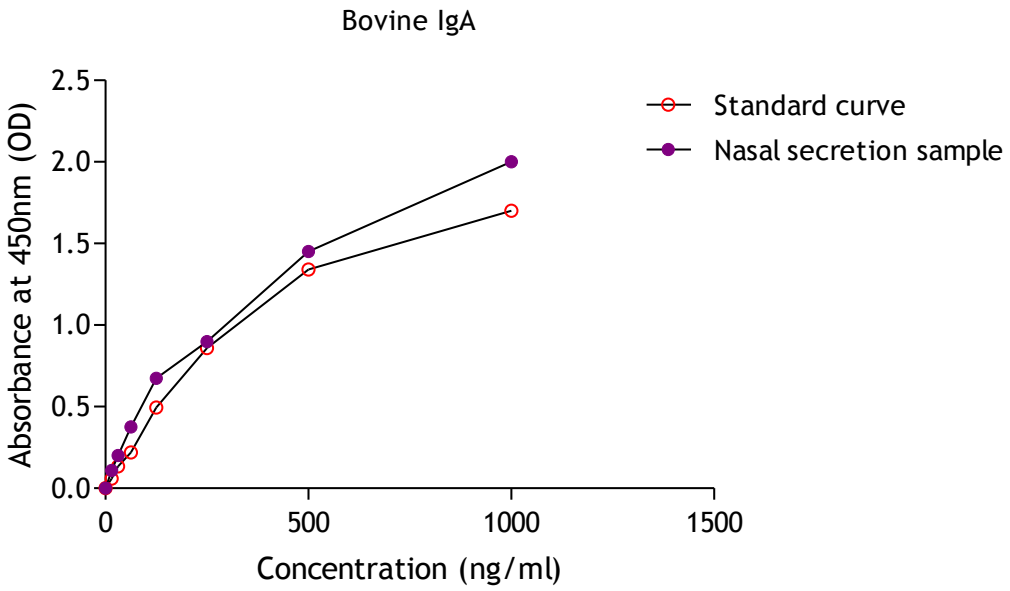


Figure 2.19: Standard curve used for analysis of A) IgA and B) IgG concentration obtained from bovine reference serum diluted at 1:100

A)



B)

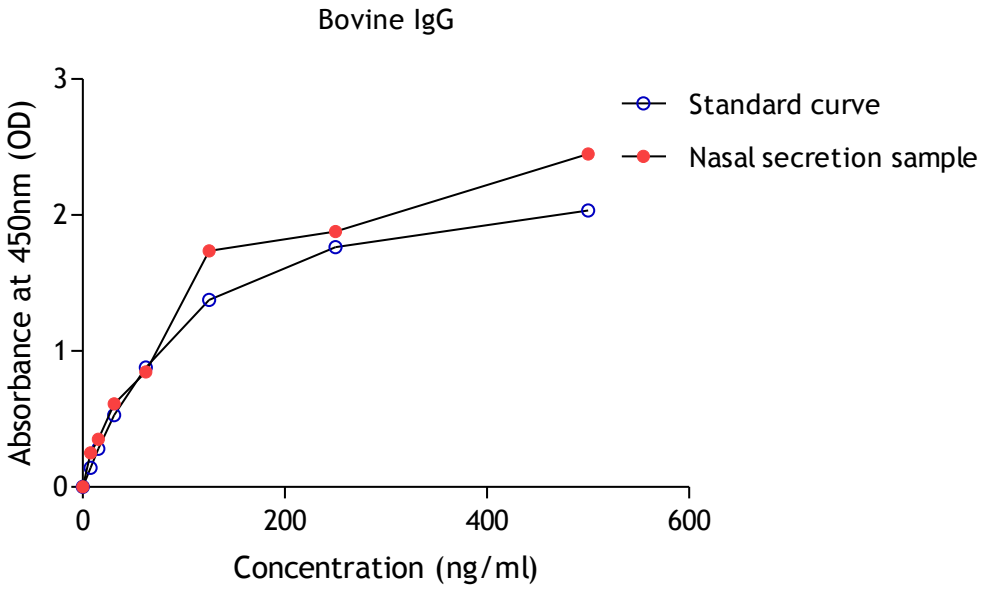


Figure 2.20: Linearity of mean NS samples at 1:2000 dilution (n=38) for analysis of A) IgA and B) IgG concentration.
Standard curve from serum diluted at 1:100 included for comparison.

2.3.7 Preliminary proteomic investigation on nasal secretion from apparently healthy cattle using one-dimensional gel electrophoresis

Ten NS samples from apparently healthy cattle were randomly selected from the 38 samples by random number generator (http://www.randomnumbergenerator.com) for preliminary proteomic investigation. Prior to one-dimensional gel electrophoresis (1-DE), the protein concentration for each sample was determined by Bradford assay and the results are presented in Table 2.4. The separation of protein from NS from healthy animals on 1-DE is shown in Figure 2.21.

Visual inspection of the polyacrylamide gel lanes revealed consistent migratory profiles between all of the samples. A total of 13 distinct protein bands were visible following 1-DE separation and were labelled a to k as shown in Figure 2.21. Apart from band a to c, which is only clearly visible in sample 4, 7 and 8 no clear difference in terms of presence or absence of protein bands were observed within the samples.

The identity of the bands in the NS was examined by the proteomic investigation fully described in Chapter 5. In this initial investigation protein identification was based on published Mw of protein in other biological fluids, notably bovine serum (Lavin et al., 2013) where an initial presumed identification was possible (Table 2.5). By comparison of the protein bands with MW marker, several proteins commonly present in NS were predicted. Albumin, commonly found in many body secretions, was predicted at ~65 kDa (band f), whereas immunoglobulins were predicted at around 55 kDa (band g) and 23 kDa (band k) for antibody fragments of heavy chain and light chain respectively. Further characterisation of protein separated by SDS-PAGE is described in section 5.2.8 – 5.2.10.

Nasal secretion no. (n=10)	Total protein (g/L)	Approximate amount of protein loaded for 1-DE separation (mg)
1	14	0.7
2	16	0.8
3	13	0.7
4	18	1.0
5	14	0.7
6	9	0.5
7	25	1.3
8	34	1.8
9	15	0.8
10	14	0.7
Median	14.5	
Range	9-34	

Table 2.4: Total protein concentration of NS from 10 different individual samples and concentration of protein diluted at 1/20 for 1-DE.
Median value and data range are displayed along the bottom of the table.

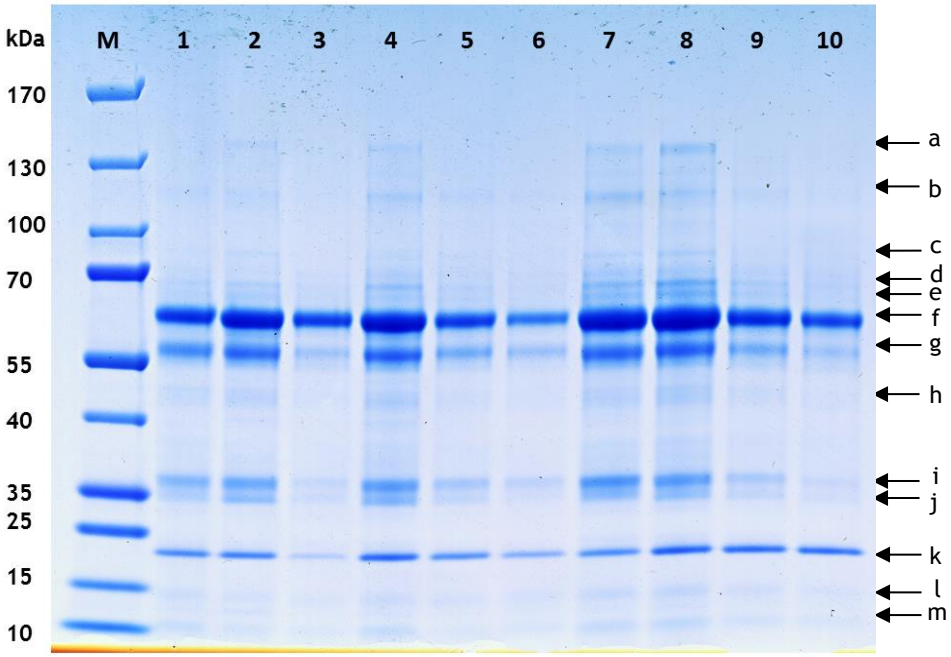


Figure 2.21: One-dimensional gel electrophoresis of bovine NS (n=10).
11 distinct protein bands were identified; each respective band is labelled (a-k). 10 µl of NS sample at 1/20 dilution were loaded in well 1-10 with approximate concentration of protein loaded correspond to Table 2.4. M= MW marker (Precision Plus Protein Standards All Blue, Bio-Rad Inc.).

Protein bands	Approximate Mw (kDa)	Presumed protein identity
a	140	n/a
b	120 - 130	n/a
c	80 - 90	*Lactoferrin*
d	68 - 70	n/a
e	67	n/a
f	65	Albumin
g	57	Ab heavy chain
h	50	n/a
i	37	n/a
j	35	*Apolipoprotein A1*
k	20	Ab light chain
l	15	n/a
m	10	n/a

Table 2.5: Predicted of abundant NS proteins in 1-DE gel based on molecular weight (Mw). Proteins in italic would be described in Chapter 2 discussion.

2.4 Discussion

Bovine respiratory disease in calves is a complex disease syndrome with many etiologic agents and is influenced by management and environment (Rice et al., 2007 and Taylor et al., 2010). The most frequent etiologic bacterial agents from fatal cases of BRD are *Pasteurellae* particularly, *M. haemolytica* (Confer, 1993; Rice et al., 2007; Katsuda et al., 2008) but, currently, there is no efficient and simple test to determine, ante mortem, the type of the disease-causing pathogen in the respiratory organs or to recognize the pathophysiological response of the animal towards this disease.

Many studies on BRD have examined the natural flora of cattle to derive an association between the recovered bacteria and disease. Some researchers sampled the upper airways of live animals (Corstvet et al., 1973), bronchoalveolar lavages (Caldow, 2001 and Senthilkumaran et al., 2013) while most studies have sampled lungs from animals that succumbed to BRD (Martin, 1983; Fulton et al., 2009; Burgess et al., 2011; and Klima et al., 2013). However, despite the likely importance of the nasal cavity as an immunologically active component of the route of infection, there has been little or no research conducted on the biochemical and immunological property of NS in cattle.

The present study investigated and characterised the biochemical profiles of bovine NS as a potential a source of information regarding the mucosal and systemic immune status of cattle. Although the study by Caldow (2001), described the technique of bronchoalveolar lavage of cattle and discussed the benefits it offers over more conventional sampling methods in the diagnosis of respiratory disease, a later study conducted in Denmark still required the use of anaesthesia to collect trans-tracheally aspirated bronchoalveolar fluid (Angen et al., 2009). Most studies have used nasopharyngeal or bronchoalveolar lavage samples for cytology and parasitology but have not obtained this fluid in an undiluted form (Allen et al., 1992; Soethout et al., 2004 and Hagberg et al., 2005). Studies on the proteomic and cellular compartments of lavage fluids required centrifugal filtration to concentrate the sample. Although nasal swabbing have been used in many investigations conducted on human and other animal species, the use of nasal swabs in cattle is mainly limited to the detection of the bacterial and viral pathogens within the lung or to determine antibiotic susceptibility (DeRosa et al., 2000; de Souza Figueiredo et al., 2010).

Prior to this investigation, there have been no studies to characterise the biochemical and immunological properties of bovine NS. However to enable using a body fluid such as NS for novel methods of detection and monitoring of disease in the cattle, it was

necessary to have a reference range of analytes that are present in the NS of healthy animal. As there is no information on the composition of bovine NS, possibly due to the perceived difficulty of collecting sufficient volume of NS for analysis, this became the first objective of the project. Swabbing the nasal tract proved to be an inefficient method of collection of the fluid. Thus a system was therefore developed in this study which allowed bovine NS to be collected in sufficient volume for multiple analyses. Previous studies have collected NS on tampons with extraction of the NS by pressure produced by a syringe (Haig, et al., 2008) but here the sample was removed from the absorbent by centrifugation using modified centrifuge and universal tubes, which allowed over 10 ml samples of NS to be collected from each animal providing sufficient volume for biochemical, immunological and, described in Chapter 5, proteomic analysis.

Biochemical analysis of the NS showed that most analytes such as urea, glucose and electrolytes were at similar concentrations to those in bovine serum, although as expected the total protein concentration was much lower. The sodium, calcium and phosphate concentration were found to be normally distributed among the NS samples. Whereas other analytes concentration such as total protein, albumin, potassium, chloride, urea, creatinine and total bilirubin were found to be not normally distributed. The variation observed may be due to individual differences present across the NS sample. The AP, AST and GGT activity were observed to be normally distributed in the bovine NS. However unexpectedly, it was found that the activities of AP and GGT were greater by factors of 15.7-fold and 2.5-fold respectively in the NS than in serum from healthy animals. There are no previous reports of the activities of these enzymes in NS being greater than those found in serum or plasma in any species. Indeed in the classic text on AP (McComb et al., 1979) there is no mention of AP in nasal mucosa or NS and only one reference to AP in olfactory cells related to the nasal cavity (Bourne, 1978). The lack of prior reports on the activity of AP in NS is likely to be due to the difficulty of collection of sufficient volumes of this fluid from animals such as laboratory rodents and reluctance of human volunteers to undertake NS collection. The two possible sources for AP in NS are due to leakage from serum, although this would require a tenfold increase in the activity by some form of concentration, or as a result of local synthesis and secretion of AP from the nasal epithelium. Elucidation of this question is the objective of Chapter 3. Alkaline phosphatase function is known in diverse biological processes, including reducing inflammatory responses in mice (van Veen et al., 2005), modulating host-bacterial interactions by dephosphorylating the Gram-negative bacterial cell wall component of LPS (Yang et al., 2013) and has been shown to detoxify this endotoxin in vitro and in vivo under physiological conditions (Poelstra et al., 1997a; Poelstra et al., 1997b and Koyama et al., 2002).

The immunoglobulins IgA and IgG, present in bovine secretions, are either produced locally by plasma cells or are derived from the blood plasma and transferred across the epithelium from the interstitial fluid compartment by selective or passive processes. It was evident from Sullivan et al. (1969) that the secretory immune system of cattle differed from that of other species where IgG was identified as the major immunoglobulin in milk, while IgA was the predominant isotype in this secretion in other species present at low concentration. However in the NS, IgA was at a higher concentration than IgG.

It has been established that a prominent local IgA system in mammals can be found in the upper reaches of the respiratory tract, with a virtual absence around the alveolar region where antibody appears to be entirely of humoral origin. In cattle and sheep, the relative abundance of IgA in the NS (Mach and Pahud, 1971 and Morgan et al., 1981) compared to IgG which prominently present in the lower respiratory tract as shown by its abundance in bronchoalveolar lavage (Pringle et al., 1988). The ELISA result conducted on NS in this study has shown that IgA has a higher concentration in comparison to IgG. This finding was also reported by Morgan et al. (1981) where IgA was shown to be the major Ig in upper NS of ruminants.

The mean result for IgG in the present study is comparable with the serum reference range provided by Duncan et al. (1972), although with several NS samples showing a higher concentration in comparison with the upper end of the reference range. There is evidence from other non-ruminant species that the proportion of IgG increases in secretions collected from lower regions of the respiratory tract (Waldman et al., 1973; Smith et al., 1975 and Morgan et al., 1980). A study conducted on ovine bronchoalveolar secretions suggested that ruminants also conform to this pattern (Gorin et al., 1979). Butler et al. (1972) have shown the concentration of IgA exceeds that of IgG in the NS, similar findings as in the present study. This would suggest that different lines of plasma cells differ in their relative proportions in tissue, in their rate of Ig production, or that mucosal IgG plasma cells contributed to the serum pool.

Both mucosal immunoglobulins, IgA and IgG share a number of properties. Immunoglobulin A is relatively resistant to proteolysis (Newby and Bourne, 1976). Both of these immunoglobulins are capable of neutralizing viruses (Smith et al., 1976 and Mazanec et al., 1992) and prevent bacterial adhesion (Fubara and Freter, 1972). Although IgA may able to reduce antigen absorption (Stokes et al., 1975), it is not a function unique to this isotype and might also be affected by IgG (Walker et al., 1973). The difference between IgA and IgG lies in their ability to fix complement.

Immunoglobulin A does not bind and interact with complement (Knop and Rowley, 1974) and it has been proposed that the consequence of failure of IgA antibody-antigen complexes to initiate hypersensitivity reaction is important in the maintenance of mucosal homeostasis (Chatel et al., 2001). Many studies have shown that IgA and IgG plasma cells occur in relatively large numbers in the respiratory and digestive system. The increase in the number of these cells (Curtain and Anderson, 1971 and Allan et al., 1979) in bovine respiratory and enteric diseases (Williams et al., 1975) suggests that these cells make a significant contribution to the humoral protection of the animal against invading pathogens.

The GGT activity found in all of the NS analysed was observed to be up to 2.5 fold higher in comparison to normal GGT values in serum reference samples. This enzyme catalyses the transfer of the gamma-glutamyl moiety of glutathione (L-gamma-glutamyl-L-cysteinyl-glycine, GSH) to an acceptor and plays a key role in the gamma-glutamyl cycle, a pathway for the synthesis and degradation of glutathione and drug and xenobiotic detoxification (Courtay et al., 1992). Consequently, GSH is a sulfhydryl-containing tripeptide that is produced by most mammalian cells and is an efficient scavenger of toxic oxidants, including hydrogen peroxide, an oxidant that plays a major role in the oxidation present on the epithelial surface of the respiratory tract in human (Cantin et al., 1989). Cantin et al. (1989) found that GSH is present in the epithelial lining fluid of the normal lower respiratory tract, where it is believed to play a major role in providing antioxidant protection to the epithelial cells. In this regard, it is possible that the main function of GGT seen in bovine NS is as a component in the pathway leading to the production of GSH, which serves to maintain integrity in the face of oxidising stressors in the respiratory tract.

Bradford analysis demonstrated that protein concentration in bovine NS was between 10 -20 g/L; almost half of the protein concentration found in bovine serum. Casado et al. (2005) reported that NS proteins present in humans are derived from local serous and mucus gland secretion and plasma exudation from the surrounding blood vessels. Therefore, the volume, concentration and types of NS protein in the cattle are dependent on the rate of glandular cell exocytosis and responses of local innate immunity towards any foreign irritation. The result of protein separation with 1-DE has shown that 13 types of protein with differing molecular weights were identified. Although the proteins were further characterised with mass spectrometry and described in Chapter 5, preliminary prediction of the abundant proteins based on Mw was possible. Mucosal proteins including albumin, having a Mw around 65-70 kDa (ALBU_BOVIN) and immunoglobulins were abundant in bovine NS. Antibody fragments of

heavy (O46780_BOVIN) and light chain (Q1RMN8_BOVIN) can be predicted to be present on 1-DE gel at around 55 kDa and 23 kDa respectively. In addition, lactoferrin, which has antimicrobial activity, had been previously reported to be present in NS (Raphael et al., 1989 and Cole et al., 1999) was and we predict that this protein is represented by a band at around 80 kDa (TRFL_BOVIN). Recently, many studies have shown the present of apolipoproteins A1 (ApoA-1) to be present in human NS (Casado et al., 2005; Mörtstedt, 2014 and Tomazic et al., 2014). Apolipoproteins are plasma proteins that are mainly involved in lipid metabolism, but also have antimicrobial and anti-inflammatory properties. Bovine ApoA-1 have a molecular weight of 27 – 35 kDa (Manjunanth et al., 1989 and Ohnsorg et al., 2011) hence it was predicted that this protein would be represented on the 1-DE gel by a band at around this Mw region.

The most striking finding of this chapter is that the enzymes AP and GGT are present in high concentrations in bovine NS and as AP has now been shown to may play a role in innate immunity, interacting in particular with LPS, there is the intriguing possibility that AP in the nasal passages has an effect on pathogens. Moreover, from this novel discovery AP may prove to be a new marker for response of the host mucosal immunity towards invading pathogen, and further characterization on this enzyme may provide better understanding on the pathophysiological response towards respiratory diseases. The next chapter will elucidate the source of AP in NS and examine the levels of AP activity in other tissues.

Chapter 3

Characterization of alkaline phosphatase in bovine nasal mucosa and comparison with other bovine tissue

3.1 Introduction

Alkaline phosphatase (AP, EC 3.1.3.1) is an enzyme that is widely distributed in nature. It has been found in blue-green algae, bacteria, and in the tissues of all members of the animal kingdom (McComb et al., 1979). Its exact function within cells is unknown however; *in vitro* it cleaves, with a high degree of specificity, the monophosphoric ester bond from a wide variety of substrates releasing phosphate radicals (Lad et al., 2003). In human, Harris (1989) and Fishman (1990) have reported at least four genes coding for AP and have been identified: non-specific AP liver/bone/kidney (*ALPL*), intestinal (*ALPI*), placental (*ALPP*) and germ cell AP (*ALPP2*) whereas in cattle, searches in NCBI database and literature have found only 2 genes coding for these enzymes which are the bovine *ALPL* (NM_176858) coding for a non-tissue specific AP and *ALPI* (NM_173987) coding for AP secreted in the intestine (Garattini et al., 1987; Hsu et al., 1987; Harris, 1989; Hahnel et al., 1990; MacGregor et al., 1995; Hoshi et al. 1997 and McDougall et al. 1998).

Alkaline phosphatase has been studied and characterized using several techniques in many species including cattle (Healy, 1971; Antonov et al., 1983; Van Hoof et al., 1988; Horney et al., 1992; Itoh et al., 2002 and Dziedziejko et al., 2009). In 1933, Graham and Kay initially demonstrated that AP activity in bovine milk was heat labile and that AP measurements could be used to monitor the effectiveness of pasteurization where AP is inactivated at a temperature that is slightly higher than normal pasteurization conditions at 65°C (Chaudhuri et al., 2013). This first method described for AP used beta glycerophosphate as substrate followed by measurement of the liberated phosphorus. A more sensitive assay using phenyl phosphate as substrate was developed by Kay and Graham (1935). In this assay, AP cleaves off phosphorus from phenyl phosphate and leaves free phenol and phosphorus in solution. The liberated phenol was measured by means of Folin and Ciocalteu colour reagent (FCR).

In 1938, Scharer improved the phenyl phosphate procedure by measuring the liberated phenol using Gibb's reagent (BQC, 2, 6-dibromoquinone-4-chloroimimide) after protein precipitation of the reaction mixture with lead acetate. Improved sensitivity was obtained when Scharer reported on the use of n-butanol extraction to remove the BQC-phenol complex from the reaction mixture (Scharer, 1938). The use of butanol extraction enabled the assay to be read visually. An AP assay using p-nitrophenyl phosphate (PNP) as a substrate has been described by Aschaffenburg and Mullen (1949). P-nitrophenyl phosphate has the advantage over phenyl phosphate since it is self-indicating. Self-indicating substrates do not require secondary colour reactions in order

to measure the product formed. Para-nitro-phenol phosphate is colourless to pale yellow in alkaline solution, but when the AP cleaves off the phosphate radical, it produces p-nitro-phenol which is deep yellow in colour with an absorbance maximum of 405 nm.

Cellular localization of target substances by histochemistry is a widely used technique in research and in histopathological diagnosis (Bitensky and Cohen, 1965 and McDougall et al., 2002). Histochemical methods have been use to localize AP in various tissue (Sugimura and Mizutani, 1979; Bell and Williams, 1979; Miao and Scutt, 2002 and Sabatakou et al., 2007). Qualitative evaluation of AP histochemistry has, for example, been used for the investigation of regulatory processes in bovine tissues (Sinowatz et al., 1982 and Van Noorden and Jonges, 1987). However, there appears to be no evidence in the literature about attempts of revealing histochemically the distribution of AP in bovine nasal mucosa.

For enzyme histochemistry, it is essential not to inactivate an appreciable proportion of the enzyme to be studied. Thus it is not enough to ensure that not more than a certain percentage of the enzyme has been lost, since it cannot be relied on that this loss occurs uniformly under all circumstances and does not represent the total enzymatic activity of a particular site within the cell (Shnitka and Seligman, 1961). This problem is most critical when studying the localization of 'soluble' enzymes, such as AP. Although Barka and Anderson (1963) contend that any histochemical demonstration of AP must involve the sacrifice of some of the enzyme by fixation in cold formalin followed by freeze-sectioning, this method has resulted in as much as 75% inactivation of this enzyme. Whereas sample fixation in acetone and embedding in paraffin wax have resulted in 80% loss of activity of AP (Barka and Anderson, 1963). Although tissue fixation is required for high quality tissue morphology, the process usually has an inhibitory effect on most of the enzymes' activity such as in AP. Snap freezing with liquid nitrogen has yielded very valuable results for the localization of AP, while maintaining tissue integrity. This method of detection (Ermert et al., 2001 and Torres et al., 2007) can provide a sensitive, high-resolution localization of endogenous AP activity in the nasal tissue hence it was the method used in this study.

Many different biochemical and immunological methods have been used to discriminate between the different AP at the enzyme and protein level. In this study, electrophoresis and immunological methods including western blot were used in attempts to characterize the AP present in bovine NS. The use of electrophoresis to separate AP isoenzymes has been reported in several species (Kaplan and Rogers, 1969; Cleeve and

Tua, 1984; Griffiths and Black, 1987 and Sharp et al., 2007). Separation and quantitation of the AP isoenzymes has commonly involved electrophoretic methods that rely on differences in their physicochemical properties (Van Hoof and De Broe, 1994). Of these, the isoelectric focusing (IEF) method has proved to be very useful and capable of separating more than 12 clinically defined AP bands (Griffiths and Black, 1987 and Griffiths, 1989).

The major function of bovine AP has been well documented in the literature summarized in section 1.3. It is believed that the report in Chapter 2 of the discovery of a high level of AP activity in bovine NS (Ghazali et al., 2014) was the first such report. In this chapter, investigations into the source and characteristics of NS AP are described.

3.1.1 Aims of study

The discovery of high AP activity in bovine NS from Chapter 2 requires further investigation regarding the source and molecular characterisation of AP in this secretion. Hence, the specific aims of the work which is presented in this chapter were to determine:

1. The source of AP in NS by localising the distribution of AP in the nasal mucosa by histochemistry.

2. To compare the AP activity level in nasal mucosa with other tissues known to produce AP by extraction and analysis.

3. To detect and quantify AP protein concentration in nasal mucosa and NS.

4. To characterize the isoforms of nasal mucosa AP by electrophoresis, western blot and IEF.

3.2 Materials and methods

3.2.1 Histochemistry

3.2.1.1 Sample collection

Bovine nasal mucosa was collected for cell morphological examination by gently separating the mucosal layer from the underlying nasal conchae, dissecting out a 1 cm square and fixing in 10% buffered neutral formaldehyde (Appendix 3.1) for at least 3 days to ensure adequate fixation. Nasal mucosa collected for localisation of AP activity by histochemistry was snap frozen in liquid nitrogen and was transferred immediately to a -80°C freezer.

3.2.1.2 Preparation of paraffin embedded tissues

Following fixation in formalin, nasal mucosa was processed using the Shandon Excelsior™ (Thermo Scientific, UK) tissue processor on a pre-set programme for 17 hours. The processed tissues were embedded in paraffin wax (TCS Biosciences Ltd, Buckingham, UK). The paraffin-embedded tissues were cut at 7 µm with a microtome (Shandon™ Finesse, Thermo Scientific, UK) and mounted onto glass slides (Superfrost™, Thermo Scientific, UK). The slides were dried at 60°C for one hour and were heated at 37°C overnight. The slides were used for cell morphological examination.

3.2.1.3 Cryosection preparation

Nasal mucosa was frozen in optimal cutting temperature (OCT) compound in a suitable tissue mould. OCT containing the tissue was freeze at -20°C onto a specialized metal grid that fit onto the cryostat. Nasal mucosa was sectioned at 8 µm thick in the cryostat at -20°C. Within 1 min of cutting a tissue section, the section was transferred to a room temperature microscope slide by touching the slide to the tissue. This step must be accomplished within 1 min of cutting the section to avoid freeze-drying of the tissue. The slide with the tissue section was immediately immersed into ethanol for fixation. The cryosection slides were stained with AP specific stain to localise the distribution of AP in nasal mucosa (section 3.2.1.5).

3.2.1.4 Haematoxylin and eosin

All sections were routinely stained with haematoxylin and eosin (H&E) for assessment of tissue morphology. Sections were deparaffinised by immersion in histo-clear (National

Diagnostics, New Jersey, USA) and then rehydrated with 70% absolute alcohol and 70% methylated spirit (Appendix 3.2) before rinsing in water. The sections were immersed in Gill's haematoxylin for five min and rinsed in water for 30 sec. Sections were dipped in 1% acid alcohol (Appendix 3.3) for 10 sec to eliminate excess haematoxylin and to enhance cellular differentiation, followed by washing in running tap water. Sections were blued in Scott's tap water substitute (Appendix 3.4) and counterstained in eosin for five min. Sections were dehydrated with 70% methylated and 70% alcohol spirit before being cleared in histo-clear and mounted with DPX (Cellpath, UK).

3.2.1.5 Alkaline phosphatase staining

Cryosections were stained with Vector® Red Alkaline Phosphatase Substrate Kit (Vector Labs, Peterborough, UK) following the manufacturer's recommended procedure (Appendix 3.5), in the presence or absence of levamisole inhibitor (Sigma-Chem Co, Poole, UK), counterstained with Gills Haematoxylin (Sigma-Chem Co, Poole, UK) and mounted with Histomount (National Diagnostics, New Jersey, USA).

3.2.1.6 Image viewing and capture

All H&E and AP stained sections were examined under a light microscope (Olympus BX51M, Southend-on-Sea, UK). Images were taken with Olympus BX51M and attached DP71 camera (Olympus, Japan), using Cell^D software (Olympus Soft Imaging Solutions, Germany).

3.2.2 Alkaline phosphatase activity in tissue extraction

3.2.2.1 Tissue samples and preparation

Tissue samples of nasal mucosa, small intestine, heart, liver and kidney samples from 6 adult cattle were collected at a slaughterhouse. Nasal mucosa was gently separated from the nasal conchae using a forceps and blade. Intestinal mucosa was obtained by gently scraping the mucosa of the small intestine with a metal spatula. Tissues were snap-frozen in liquid nitrogen and stored at -80°C prior to extraction.

3.2.2.2 Butanol protein extraction

Butanol AP extraction was conducted according to the method of Rudolph et al. (1997). Briefly, 2 g from each tissue were washed with isotonic saline solution and homogenized (Janke & Kunkel Ultra Turrax T25) in 5 ml saline. Alkaline phosphatase was extracted

from 2 samples of crushed cancellous bone obtained from the metacarpal bones of 2 different cattle.

Tissue AP extracts were further processed by incubation with 100% n-butanol in ratio of 1:4 of n-butanol:protein homogenates, with gentle agitation at 4°C for 30 min. Extraction with n-butanol facilitates the release of AP from membranes where it is anchored to phosphatidylinositol. The samples were centrifuged at 1,500 × *g* for 30 min at 4°C, the aqueous layer removed and centrifuged at 15,000 × *g* for 90 min with the supernatant being the AP extract.

3.2.2.3 Measurement of alkaline phosphatase activity

The AP activity and the protein concentration in the AP extracts were determined as described in section 2.2.5.1. The AP activity of tissue extracts was recorded as enzyme activity per gram of tissue extracted (IU/g).

3.2.2.4 Statistical Analysis

Statistical analyses and graphs were performed using GraphPad Prism for Windows, version 5.0 (GraphPad Software Inc., California, USA). The normality of the data was examined using the D'Agostino and Pearson Omnibus normality test. The relative AP activity in all different extracts was compared using Kruskal-Wallis test and individual pair comparison using Dunn's Multiple Comparison Test. Significance level (α) was set at 0.05 for both tests.

3.2.3 Western blot analysis

3.2.3.1 Sample preparation

Nasal mucosa and liver extraction from 6 different cattle carried out as in section 3.2.2.1 were used for this experiment. Samples were diluted with ultrapure water to a concentration of 2-3 mg/ml. Laemmli sample buffer (Bio-Rad Laboratories, Hemel Hempstead, UK)) and ß-mercaptoethanol were added into the samples and were denatured by heating at 95ºC for 4 min.

3.2.3.2 Antibody and conjugation

Monoclonal antibody raised in mouse against human bone AP (Abcam, Cambridge #ab 17272) was used to detect AP in the tissue extraction. Conjugation of anti-bone AP

antibody with horse radish peroxidase (HRP) was performed using Lightning Link™ HRP conjugation Kit (Innova Biosciences, Cambridge #701-0030) according to manufacturer's instructions. Antibody used in this experiment were diluted with 5% skimmed milk at a dilution of 1/2000 (Anti-Bone AP conjugated with Lightning Link™ HRP).

3.2.3.3 Western blot

One-dimensional electrophoresis was carried out as in section 2.2.8. Proteins were transferred to a nitrocellulose membrane using the Mini Trans-Blot® Electrophoresis Transfer cell (BioRad, Hamel Hempstead) in 1x transfer buffer (Appendix 3.6). Fifteen µl (25 µg) of sample was loaded into each SDS-PAGE well and electrophoresis separation was conducted at 300 V for 20 min. The electrophoretic transfer was performed at 70 V for 60 min at 4ºC. Once proteins were transferred onto the nitrocellulose membrane, the membrane was blocked in 5% skimmed milk (Appendix 3.7) for 60 min. Membrane blots were then incubated with anti-bone AP conjugated to HRP antibody (section 3.2.3.2) at a dilution of 1/2000 for 2 hr. Later, membrane blot was washed 3 × 5 min in TBST (Appendix 3.8).

3.2.3.4 Image acquisition and assessment

Visualization of immunoreactivity was completed using Pierce ECL to detect HRP activity (Thermo Scientific, Paisley #32209) according to the manufacturer's instructions. Membranes were wrapped in Saran Wrap and were then exposed to radiographic film for between 30 sec and 5 min. Radiographic films were scanned using a UMAX PowerLook III Scanner (UMAX Technologies Inc, California, USA) under the following settings: transmissive mode, grey scale and a resolution of 600 dpi

3.2.4 Agarose gel electrophoresis for alkaline phosphatase isoenzyme detection

Samples of bovine NS and tissue extracts from NS, nasal mucosa, heart, small intestine, liver, kidney and bone were selected from the available samples for the separation of AP isozymes based on electrophoretic mobility on agarose gel. Samples were diluted to an AP activity of 280-350 IU/L with water and run on 2% agarose gel (Appendix 3.9). A total of 15 µl of tissue extract was subjected to electrophoresis through a horizontal agarose gel in 1 × TAE buffer pH 8.6 for 45 min at 250 V. The agarose gel was then stained with Pierce 1-Step™ NBT-BCIP AP substrate solutions (Thermo Fisher Scientific Inc, Illinois, USA) for up to 1 hr at 37°C. Gel Images were acquired using UMAX

PowerLook III Scanner (UMAX Technologies Inc., California, USA) under the following settings: transmissive mode, true colour RGB and a resolution of 600 dpi.

3.2.5 Isoelectric focusing

Samples of bovine NS and tissue extracts from NS, nasal mucosa, small intestine, liver, kidney and bone were selected from the available samples for the separation of AP isozymes based on pIs. Samples were diluted to an AP activity of 280-350 IU/L in IEF sample buffer pH 3-7 (Life Technologies, Paisley, UK) prepared according to manufacturer's instructions. The pI of the various AP isozymes was determined by separation using Invitrogen Novex® pH 3–7 IEF Gel (Life Technologies, Paisley, UK) and comparison to IEF standards. Ten µl of the prepared sample were loaded into each well such that IU of AP were loaded per well. Isoelectric focusing was conducted at 4°C using the following voltage gradient: 100 V, 1 hour; 200 V, 1 hour; 500 V, 30 min. The gel was then stained with Pierce 1-Step™ NBT-BCIP ALP substrate solutions (Thermo Fisher Scientific Inc, Illinois, USA) for 5 hours at 37°C. The pI of focussed bands was estimated in comparison to pH3-10 standards (Serva Electrophoresis, Heidelberg, Germany) which were run in a track of the IEF gels and stained with Coomassie blue following focusing and destained in 10% (w/v) acetic acid in 25% (w/v) methanol for 24 hours. Gel Images were acquired as described in section 3.2.4.

3.3 Results

3.3.1 The distribution and localisation of AP activity

Histological assessment of bovine nasal mucosa stained with H&E show the respiratory portion of the nasal mucosa is lined by a ciliated, pseudostratified columnar epithelium with goblet cells. The lamina propria contains tubuloralveolar glands mainly serous and mucous glands (Figure 3.1 left columns). Histochemistry using an AP activity stain (Vector Red substrate) showed strong AP activity in the luminal and basal ends of nasal epithelium and serous glands (Figure 3.1 middle columns). Alkaline phosphatase activity staining was abolished in the presence of levamisole a known non-specific AP inhibitor (Figure 3.1 right columns).

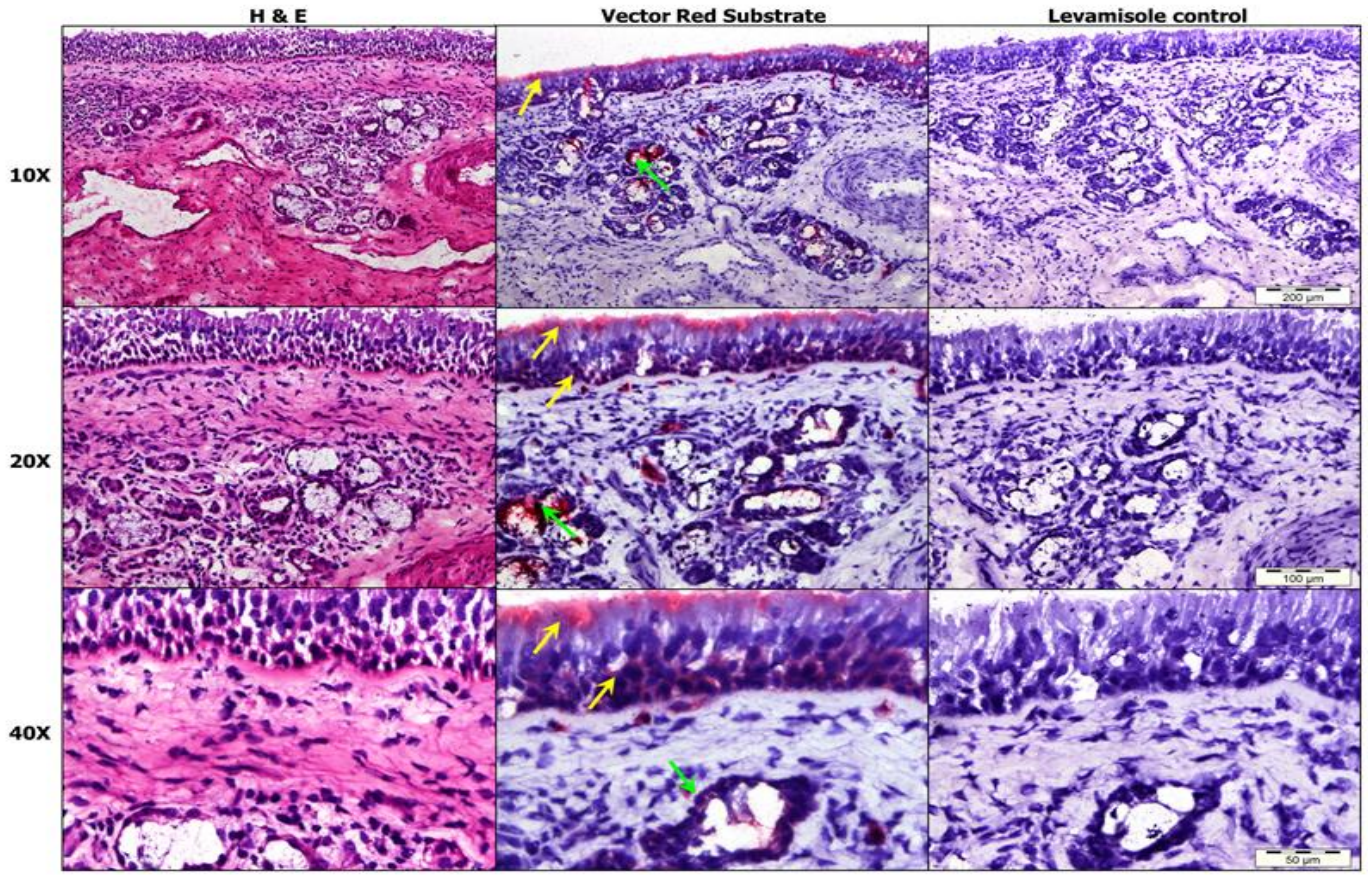


Figure 3.1: Histology and AP activity histochemistry of bovine nasal mucosa.
Routine staining of nasal mucosa with H&E (left column) and histochemical staining of AP activity using Vector Red substrate (central column) showed strong AP activity in the mucus at the luminal surface and in the basal cell layers of the epithelium (yellow arrow); as well as in serous glands (green arrow). Levamisole, a non-specific AP inhibitor, abolished the histochemical staining of these cells (right column). Image captures at x10, x20 and x40 magnifications are shown with appropriate scale bars.

3.3.2 AP activities in nasal mucosa and other tissues

The extracts from nasal mucosal tissue (n=6) had mean AP activities which were not significantly different from the mean activity extracted from other bovine tissues known to produce this enzyme: liver, intestine and kidney, although the nasal mucosa extracts did have the highest median activity per gram of tissue extracted. All of these extracts had significantly higher AP activities per gram of tissue extracted compared with the heart tissue (Table 3.1) that served as a negative control.

Statistical analysis using Kruskal-Wallis test to compare the median AP activity between the bovine tissues revealed a significant difference ($p<0.05$). However, Dunn's Multiple Comparison Test was used to compare AP activity between individual tissue pairs. The results have shown that apart from AP activity compared between heart vs. nasal mucosa and heart vs. liver, comparison of AP activity in other tissue pairs show non-significance difference ($p>0.05$). Data were presented in a box and whisker plot (Figure 3.2).

Tissue	Mean ± SD (IU/g)	Median (Range)
Nasal mucosa	14.2 ± 9.5	14.3 (1.0 - 27.6)
Intestinal mucosa	10.2 ± 8.8	6.5 (2.2 - 24.0)
Liver	11.5 ± 9.8	8.3 (1.0 – 27.2)
Kidney	8.6 ± 4.4	7.4 (3.9 - 16.2)
Heart	0.7 ± 0.3	0.6 (0.2 – 1.1)

Table 3.1: Alkaline phosphatase activity in bovine tissue extracts (n=6).

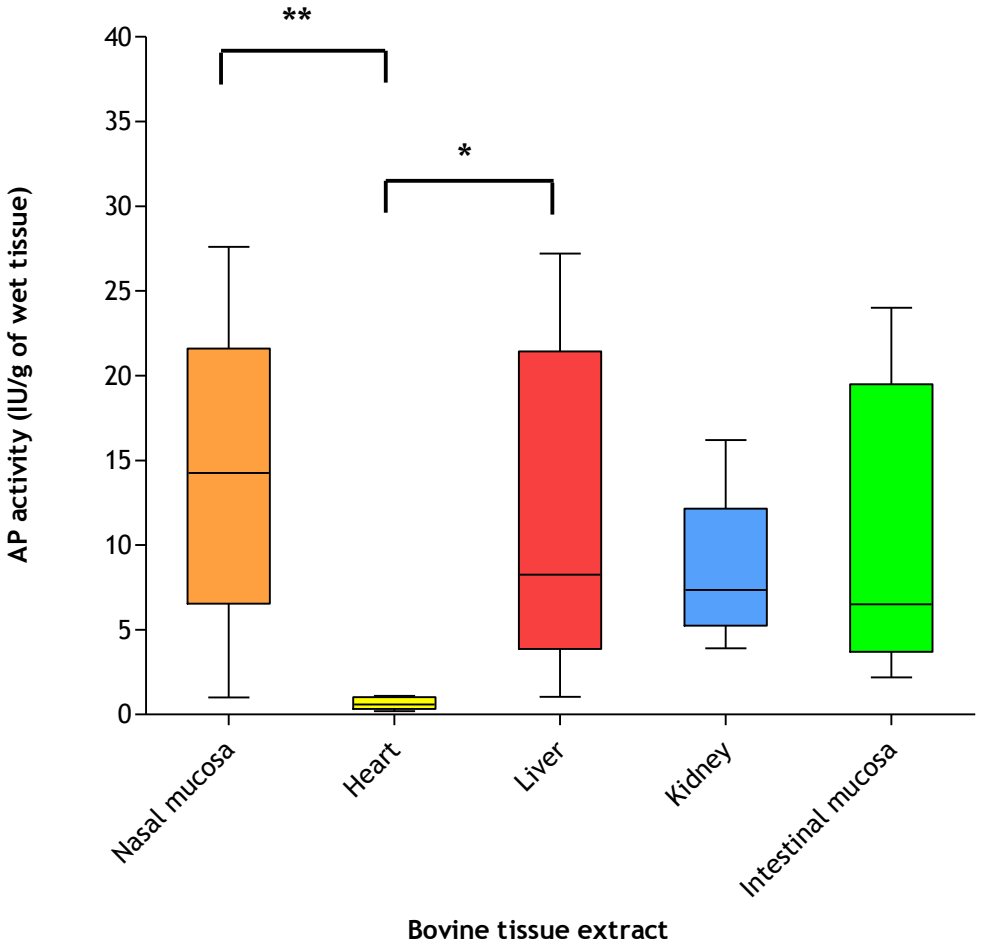


Figure 3.2: Comparative of AP activity per gram of wet tissue in various bovine tissues (n=6).
The AP activity in corresponding bovine tissue was quantified, expressed as enzyme unit per gram (IU/g) of tissue. Data presented in median with 25 to 75 percentile range. Horizontal bar indicating comparison of AP activity between tissue pairs with * and ** corresponds to significant difference of P ≤0.05 and P≤0.01 respectively.

3.3.3 Western blot analysis

3.3.3.1 Optimisation of AP antibody on nasal mucosa and liver

In order to characterise the AP isoforms present in NS and nasal mucosa, it would be valuable to investigate the enzyme using an antibody based approach. There is no antibody against bovine non-specific AP hence the monoclonal antibody against human bone AP (tissue non-specific AP) was used. A number of antisera to AP were screened for cross reactivity [polyclonal rabbit anti-human bone AP and monoclonal rat anti-human recombinant ALPL (Insight Bio., Middlesex)] with the mouse anti-human bone AP giving the greatest response in initial Western blots. Thereafter, numerous attempts to use western blot were made using a second antibody system as well as direct conjugation of antibody to reporter enzyme. The use of AP antibody as a two-step protocolwith a second antibody resulted in non-specific effect of AP antibody reacting with bovine Ig in the tissue samples. An attempt was made to use conjugation of antibody with as biotin (Thermo, Paisley #21217) however the biotinylated AP antibodies did not produce any signal. Furthermore the biotinylation method used required high volume of AP antibody and involved long incubation time to conjugate. None of these approaches gave satisfactory results where AP isoform detection could be validated to an acceptable level.

However, conjugations of antibody against human bone AP with HRP using Lightning Link™ HRP conjugation Kit (Innova Biosciences, Cambridge #701-0030) provided the clearest banding pattern on western blot. Figure 3.3 summarises the cross-reactivity of antibody against human bone AP that were conjugated with HRP in bovine nasal mucosa and liver extracts. Antibody diluted to 1/1000 showed a stronger signal compared with sample incubated in 1/2000 of antibody.

3.3.3.2 Western blot assessment

For this experiment, 10 µl (15 µg) of sample was loaded into each SDS-PAGE well for electrophoresis separation and were transferred to radiographic film as described in section 3.3.3.1. Assessment of the HRP conjugated AP antibody reaction with nasal mucosa and liver samples from different animals (n=8) demonstrated comparable signal patterns between the group (Figure 3.4). Alkaline phosphatase antibody should produce a positive signal at approximately 57 kDa. However, generally AP antibody has produced a robust signal in nasal mucosa (~70kDa) and a weaker signal at the same molecular weight was seen in the liver and other tissue extract. Additionally, for nasal mucosa

another 3 bands were detected at 35, 55 and 150 kDa in each of the individual samples. Another 5 weaker signals at approximately 25, 35, 40, 55 and 130 kDa were seen in all the liver samples. The detection of several signals in a tissue with the strongest around 70 kDa and at different molecular weights indicates that the AP antibody has cross reactivity with other proteins. It was speculated that the antibody might cross react with albumin, which has a molecular weight of 66.5 kDa, consistent with the approximately 70 kDa band.

3.3.3.3 Comparative analysis of alkaline phosphatase signals in nasal secretion and other bovine tissues.

Another investigation on the cross reactivity of AP protein was performed in other bovine tissues. Commercially prepared bovine IAP was used as positive control and commercially prepared bovine serum albumin (BSA) at the concentration of 2 mg/ml was use as a negative control. The gross protein profile for each sample was visualised by Coomassie Briliant Blue stain and demonstrated a comparable pattern between samples (Figure 3.5A). Analysis of the membrane blot showed that a robust signal was detected at approximately 70 kDa in all the samples and controls with other weaker signals detected in NS and tissue samples at various molecular weights (Figure 3.5B). A strong reaction was also obtained with commercial IAP at 65 kDa.

3.3.3.4 Influence of albumin on AP stability

This study was done to determine the effect of albumin on AP antibody reactivity and if pre-incubation with albumin would remove cross reactions. The effect of using AP antibody mixed with 4% albumin for incubation of membrane blot is shown in Figure 3.6B. Although the presence of albumin slightly reduces the signal of other proteins in all the tissues, AP antibody retained its reactivity to other proteins for example with albumin which produced the strongest signal at ~70kDa (Figure 3.6A and B).

3.3.3.5 Normalization effect

This study was conducted to analyse the response of NS and other bovine tissue extracts to AP antibody after protein concentration and AP activity were normalized before electrophoresis. The protein profiles were assessed by Coomassie Brilliant Blue and are comparable across the different samples analysed with normalization of protein at 20 µg (Figure 3.7Ai and Bi) and with samples normalized at an AP activity of 300 -350 IU (Figure 3.7Aii and Bii). There is no gross variation seen on the protein profiles as a consequence of the normalization treatment. A robust signal of approximately 70 kDa

was detected in all samples except for bone tissue extract. Several weaker signals at various molecular weights were also detected in NS and all tissue extracts.

3.3.3.6 Comparative analysis of alkaline phosphatase signals with unconjugated anti-alkaline phosphatase antibody

This study was conducted to demonstrate the effect of HRP protein conjugation on AP antibody used throughout this study. Hence unconjugated AP was used to detect AP signal in nasal mucosa and liver tissue extracts. Anti-mouse IgG antibody conjugated with HRP was used as the secondary antibody and detected by calorimetric HRP (Opti-4CN™). Results showed that AP antibody apart from showing a robust signal at approximately 70 kDa in all the samples it has also cross reactivity with several protein bands mainly seen at 30 and 160 kDa (Figure 3.8).

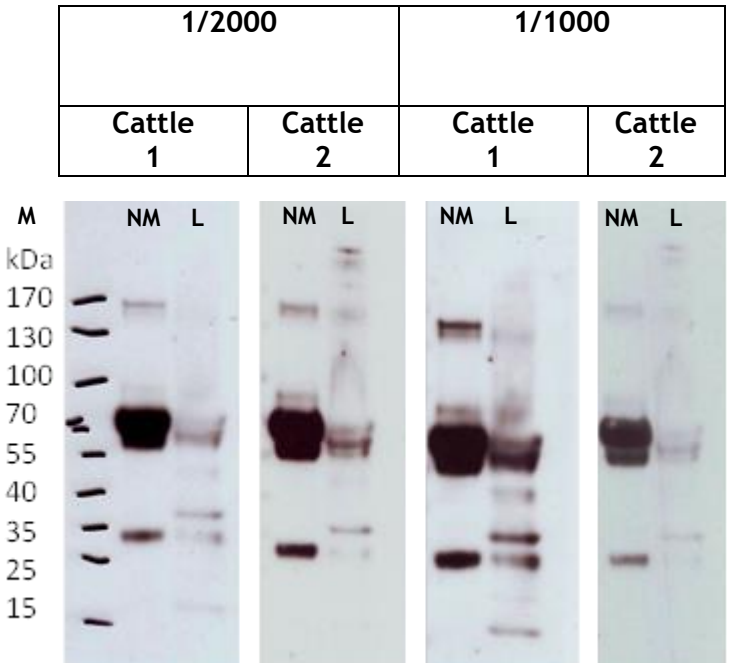


Figure 3.3: The optimisation of HRP conjugated antibody against bone AP in nasal mucosa and liver extracts (n=2).
Antibody was diluted with 5% skimmed milk at 2 different concentrations 1/1000 and 1/2000. NM=nasal mucosa; L=liver and M=protein marker.

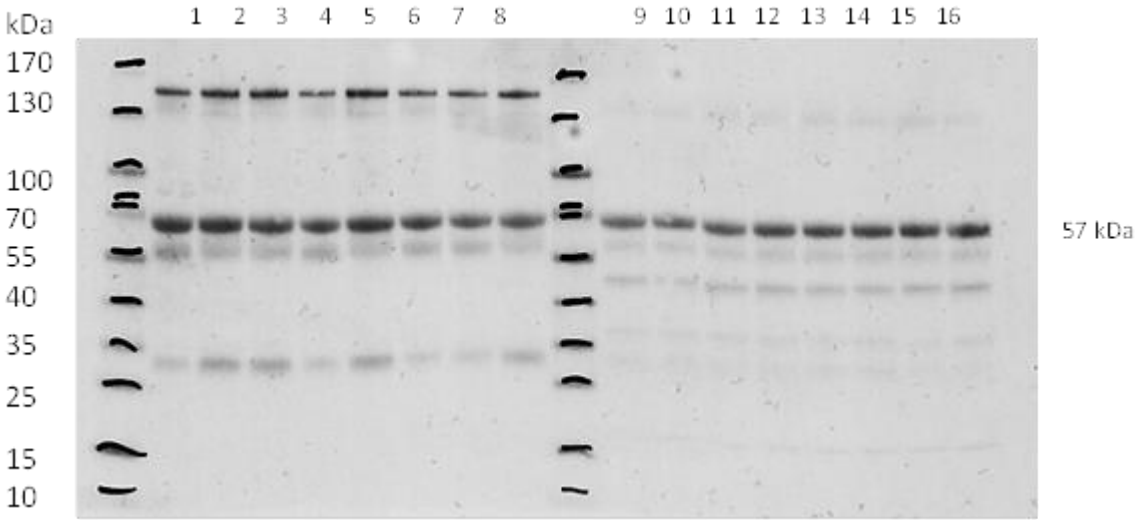


Figure 3.4: : Preliminary western blot analysis of AP in nasal mucosa and liver.
The AP signals obtained from western blot analysis in nasal mucosa (n=8; lane 1-8) and liver (n=8; lane 9-16).

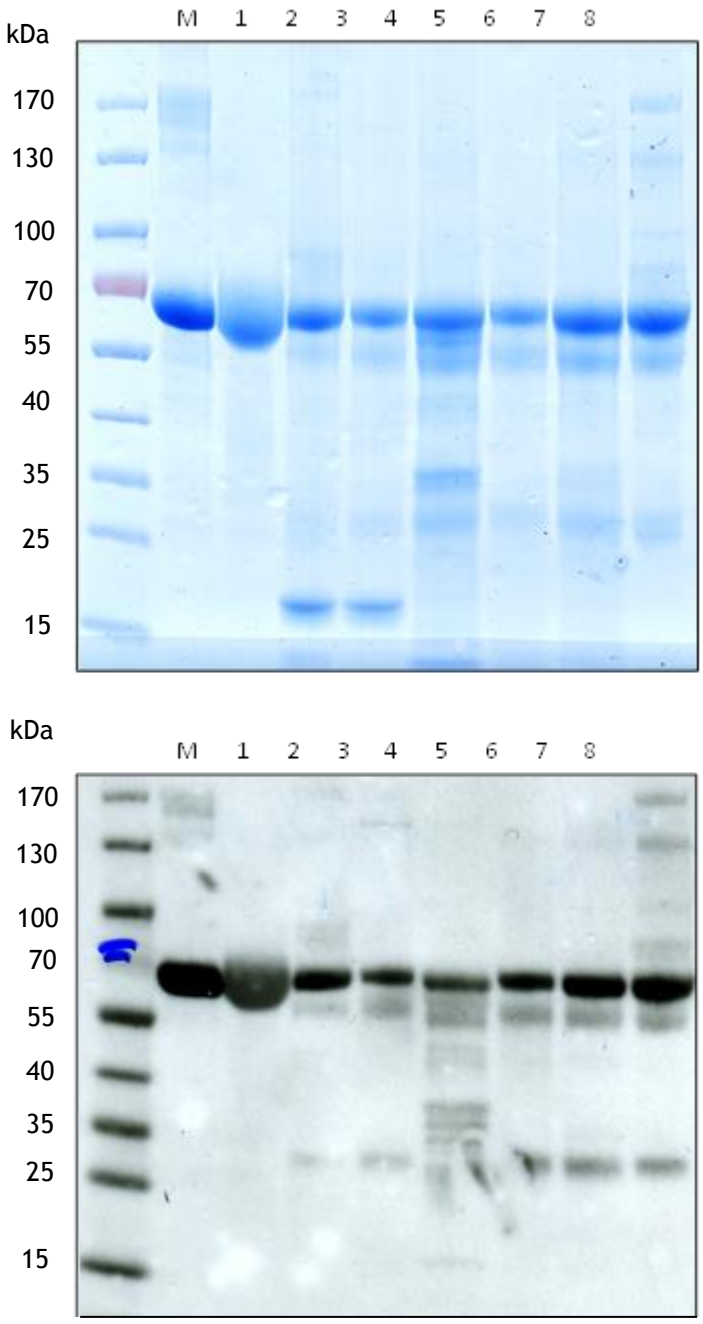


Figure 3.5: Comparative analysis of AP in NS and other bovine tissues.
A) SDS-PAGE of NS and tissue samples stained with Coomassie Brilliant Blue and subsequently compared with commercially prepared controls. B) The AP signals obtained from western blot analysis show signals for all samples and prepared controls. M=protein marker; 1=commercially prepared BSA 2mg/ml; 2=commercially prepared bovine IAP; 3=NS; 4=nasal mucosa; 5=liver; 6=intestine; 7=kidney; and 8=bovine serum 4 mg/ml.

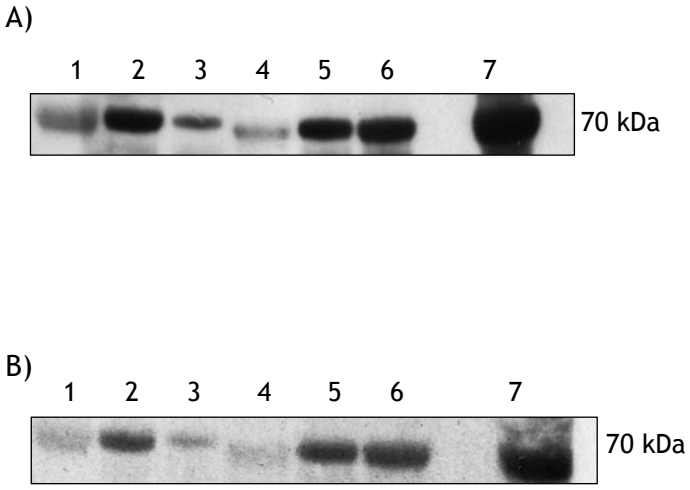


Figure 3.6: The influence of albumin on AP stability.
The protein signals obtained from western blot analysis incubated in A) 1/2000 AP LL-HRP antibody and B) 1/2000 AP LL-HRP antibody with 4% albumin. Although the albumin reduces the protein signal, AP antibody still has reactivity with other proteins at different molecular weight for example given here is albumin (~70kDa). 1=commercially prepared bovine IAP; 2=NS; 3=nasal mucosa; 4=liver; 5=intestine; 6=kidney and 7=bovine serum 4 mg/ml.

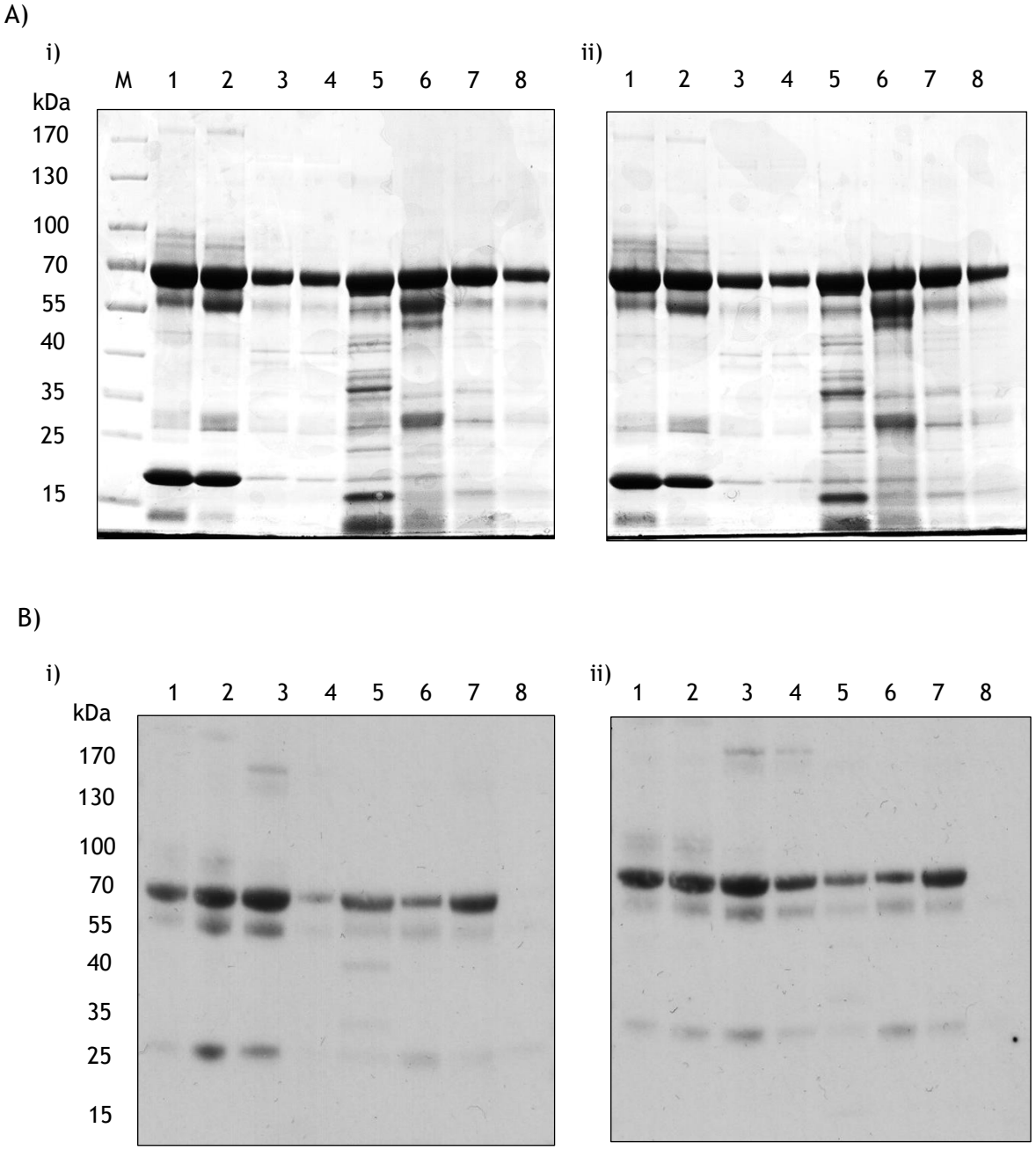


Figure 3.7: Comparative analysis of AP Western blot signal between normalization effects of i) protein concentration and ii) AP activity.
A) The protein profiles were assessed by Coomassie Brilliant Blue and are comparable across the different samples analysed with normalization of protein at 20 µg and AP activity at 300 - 350 IU. There is no gross variation seen on the protein profiles as a consequence of the normalization treatment. B) The AP signals obtained from western blot analysis show a non-specific AP signals for all samples and negative for lane 8. 1&2=NS; 3&4=nasal mucosa; 5=liver; 6=intestine; 7=kidney; 8=bone and M=protein marker.

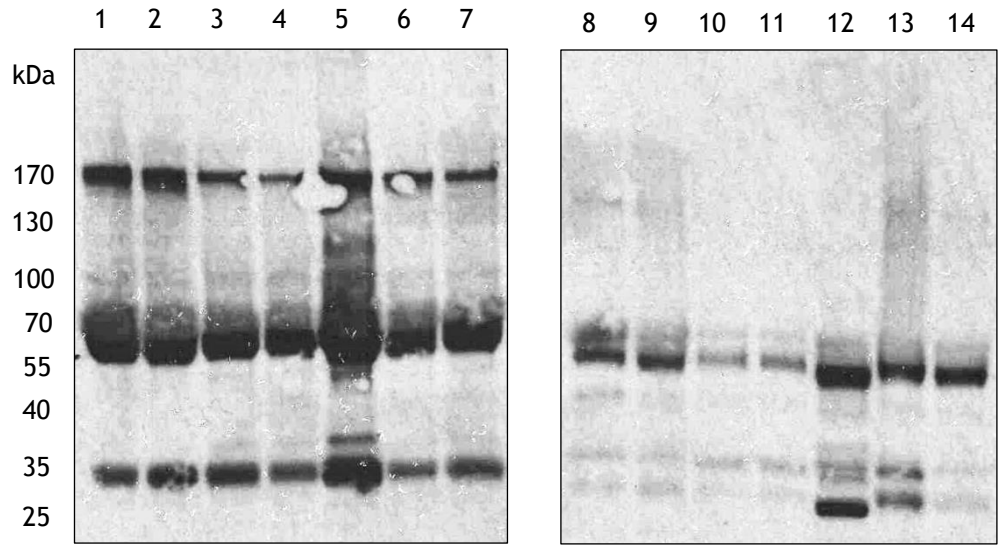


Figure 3.8: Comparative analysis of AP signals in nasal mucosa (n=7) and liver (n=7) with unconjugated AP antibody.
AP signals obtained from western blot analysis incubated in primary AP antibody at 1/2000 and secondary anti-mouse HRP in nasal mucosa (lane 1-7) and liver (lane 8-14) and detected with calorimetric HRP (Opti-4CN™).

3.3.4 Agarose gel electrophoresis

Electrophoresis of NS and various tissues extracts on an agarose gel followed by staining for AP activity produced bands for all the samples except for the bovine heart sample as expected due to its very low AP activity (Figure 3.9)

Visual assessment showed that AP from NS and nasal mucosa extracts has the same mobility on agarose. Apart from the band produced from liver extract which shows the most anodal from the origin, the AP bands from all other tissue extracts (bone, kidney and intestine) have shown having lower electrophoretic mobility than the NS and nasal mucosa extracts though the variation in their mobility seen was not clearly distinct.

3.3.5 Isoelectric focusing of alkaline phosphatase isoenzymes

The AP from various bovine tissues separated by IEF and stained by zymography with an AP substrate, are shown in Figure 3.10. Although diluted to a similar AP activity, notable differences were observed in the band intensity of the AP isoforms. Close inspection of the loading well at the cathodal end of the gel, for the NS samples showed a deposit of AP activity, suggesting that a proportion of the AP in these samples was unable to enter the polyacrylamide gel, possibly due to the formation of large aggregates.

Nevertheless, the IEF demonstrated that AP from the NS had isoforms with bands with AP activity at pH 4.8 (Figure 3.10-1) and 5.0-5.3 (Figure 3.10-2). The nasal mucosa extract also had major bands at pH 4.8-5.2 (Figure 3.10-3) and several minor bands at pH 5.3 - 6.2 (Figure 3.10-4). Bovine bone AP had an isoform at pH 4.8 (Figure 3.10-5) and 5.0-5.3 (Figure 3.11-6), as in NS with several bands at pH 6.0 – 6.7 (Figure 3.10-7). The liver AP resolved at lower pI with a major band at pI 4.6 (Figure 3.10-8). Intestinal and kidney AP had isoforms pI of 5.4-6.2 (Figure 3.10-9) with kidney AP also having major bands between at pI 5.2 and 5.3- (Figure 3.10-10). The pI of the isoforms were estimated by comparison to standard protein of known pI value run on the same IEF gel with the track of the standard proteins separated and visualised with Coomassie blue protein stain rather than with AP zymography.

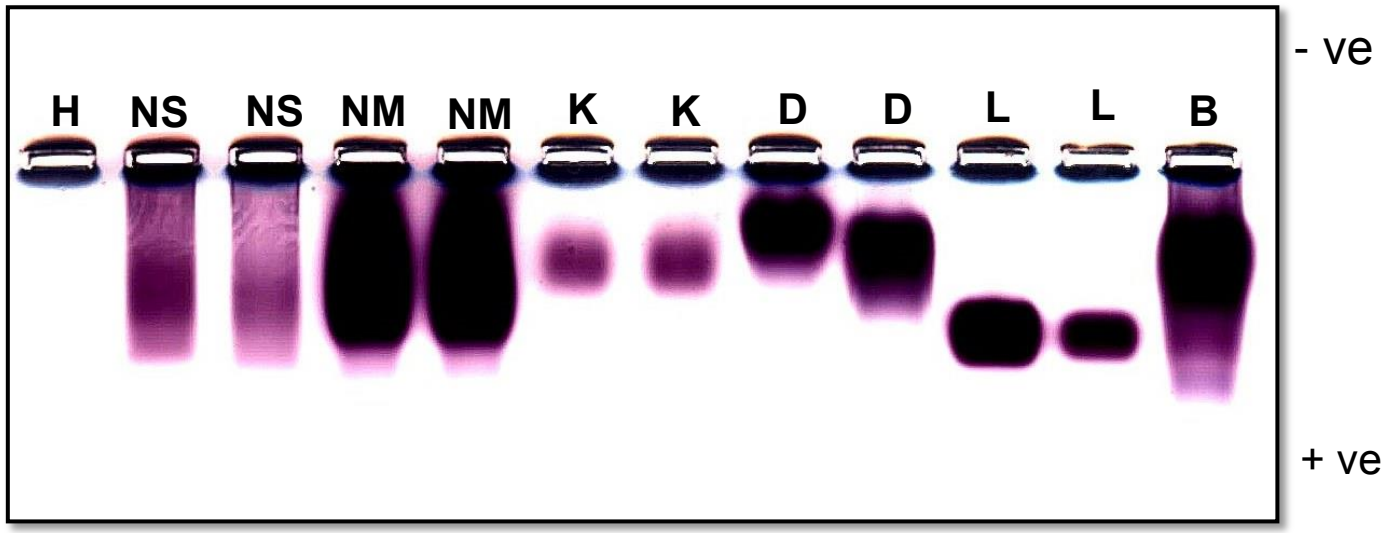


Figure 3.9: Agarose gel electrophoresis of AP in NS and bovine tissue.
Bovine tissue extracts and NS separated on 2% horizontal agarose gel stained with 5-Bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium chloride (BCIP/NBT) substrate. H = heart; NS = nasal secretion; NM = nasal mucosa; K = kidney; I = intestine; L = liver; B = bone; -ve = cathode; +ve = anode

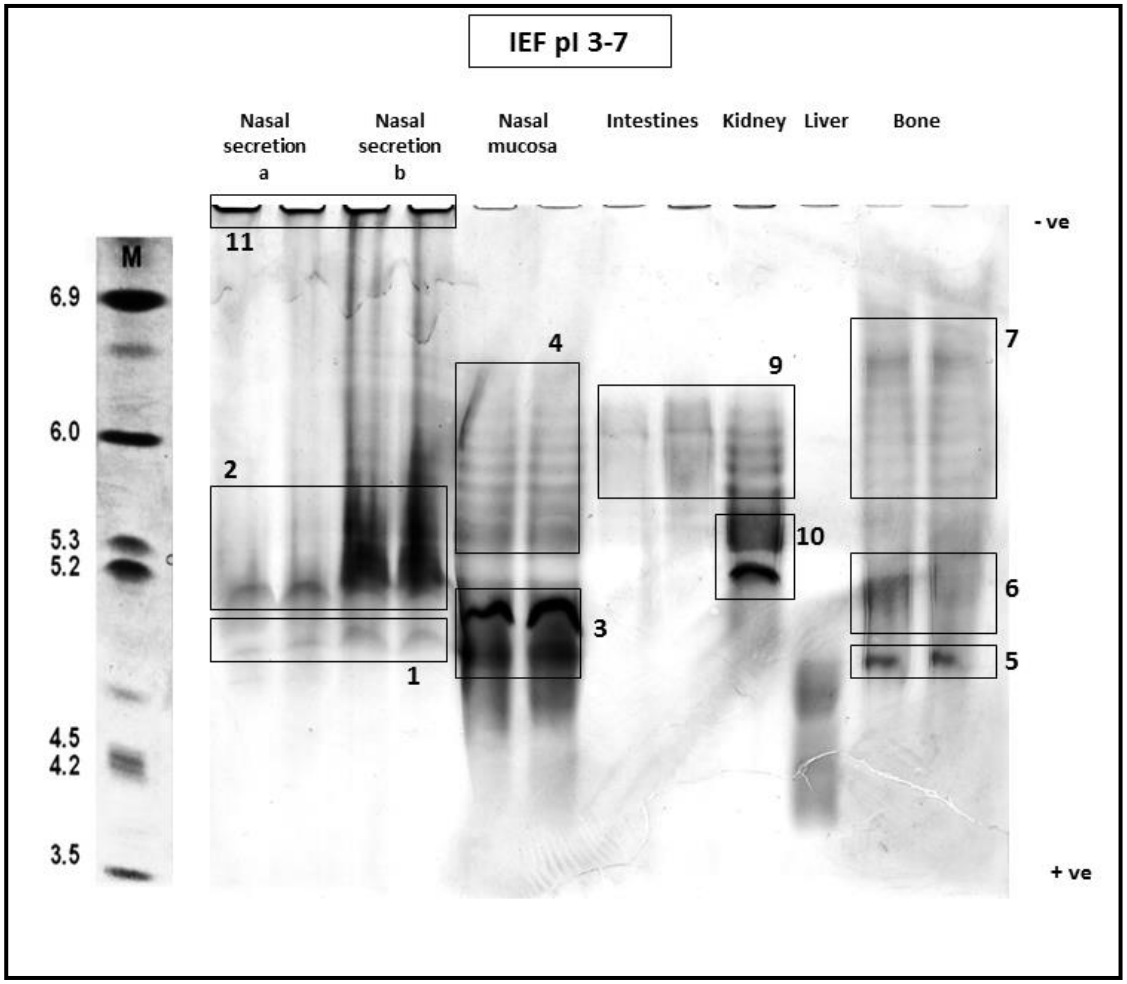


Figure 3.10: Isoelectric focusing of AP in bovine tissue and NS.
Bovine tissue extracts and NS separated on IEF gels over a pH 3–7 range and stained with 5-Bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium chloride (BCIP/NBT) substrate. M = IEF standard protein marker (stained with Coomassie blue); −ve = cathode; +ve = anode. Two different samples of NS were run in duplicate. Samples of extracts from nasal mucosa, bone and intestine were run in duplicate with liver and kidney extract being run in single tracks. Annotated boxes around isoforms bands as discussed in section 3.3.4.

3.4 Discussion

In this chapter, experiments were carried out to investigate the source of AP that was found in bovine NS and further characterize this enzyme to provide information regarding the possible role it plays in host defence or in maintaining homeostasis.

Within lung tissue, non-specific AP activity has been previously demonstrated within the cell and on the surface of respiratory epithelium in humans, where it is able to dephosphorylate ATP to AMP and to adenosine, important for mucociliary clearance (Picher et al., 2003). The enzyme was also reported several decades ago in human nasal mucosa (Bourne, 1948) and more recently described in this tissue and in micro litre volumes of NS of guinea pigs (Gawin et al., 1991) and in the related olfactory epithelium of mice and rats (Gladysheva, 2002). Apart from these reports, the activity and production of AP in NS and nasal mucosa has not, to our knowledge, been documented. However the prior identification of AP in the nasal mucosa did indicate that the AP in the NS may be due to secretion of the enzyme from the mucosal cells.

This histochemical technique for AP localization conducted in this study was found to be highly reproducible and easy to perform. Fixation was found to be a critical step in the successful demonstration of AP activity in nasal mucous tissues. Pilot histochemistry examination results show that the use of periodate-lysine-paraformaldehyde (PLP) although does not inhibit/destroy AP activity it reduced the tissue and cell constituents for morphological analysis. Snap freezing with liquid nitrogen preserves AP activity very well and assists in tissue sectioning while maintaining the tissue cell integrity. Additionally, inhibition of AP activity by levamisole provided a clear distinction of bovine isozymes in this histochemical analysis. By employing the histochemical technique for AP established in this study, the localization of AP on nasal mucosa has been confirmed. The distribution of AP activity as assessed in the present study is mainly present in the mucus at the luminal surface and in the basal cell layers of the epithelium as well as in the serous glands of the nasal mucosa as shown in Figure 3.1. The findings confirmed that NSAP was produced locally by the nasal mucosa cells and were secreted into the luminal surface by serous glands exocytosis.

The second aim was to measure and compare the AP activity level in nasal mucosa with other relevant tissue known to produce AP: liver, intestine and kidney. The AP extraction method used was similar to a method described by Rudolph et al. (1997) and results expressed in enzyme activity/g of wet tissue used. The AP derived from the nasal mucosa extracts (n=6) were similar in activity to those of liver, kidney and

intestinal mucosa. However, nasal tissue and liver had significantly higher activities than heart. This level of AP activity in nasal mucosa justifies further investigation on its function in bovine NS. The finding of AP activity being higher in nasal mucosa compared with serum AP reference values also points to the source of AP in NS as being of nasal mucosal origin and secreted locally rather than due to plasma leakages. Comparison of AP activity with bone extracts was not included in this study due to the lack of fresh bone samples. It would be important to include bone tissue in future study of AP activity due to its vital role in calcium homeostasis and in skeletal mineralization.

However, the variation of AP activity observed in nasal mucosa and other bovine tissues do not represent the actual concentration of the AP enzyme present in these tissues. Hence, the next aim was to detect and possibly quantify the AP concentration in nasal mucosa and other relevant tissue by Western blotting. This method could also be used to validate AP antibody for future work on NSAP such as immunocytochemistry. Western blot analysis to determine the specificity of AP antibody used was carried out in NS and in various tissue extracts and included BSA and commercially prepared bovine IAP as negative and positive control respectively. It was found that apart from the possible detection of AP as a band of approximately 57 kDa, a stronger band at a molecular weight of approximately 70 kDa was also detected in all the samples (Figure 3.4). A band at approximately 70 kDa was also present in BSA and IAP with the former indicating that the antibody used in this study was not specific for AP protein. It was inferred that the antibody cross reacts with albumin based on the finding that antibody detects the commercially prepared BSA and detected bands in all samples at the molecular weight similar to bovine albumin (~70 kDa).

The apparent cross-reactivity with numerous proteins could be due to possible intrinsic characteristics of the BSA with AP or the non-specific effect of the AP antibody reacting with bovine immunoglobulins. However, the reaction with commercial IAP and the presence of some bands on western blot not visible in Coomassie blue stained gels may indicate that the antibody has some reaction with bovine APs. However based on the poor results demonstrated by Western blotting and the cross reactivity of the AP antibody (Abcam, Cambridge #ab17272) against albumin and other high abundance proteins present in NS, tissue extracts and controls used in this study, it was concluded that this approach with this antiserum would be unlikely to yield reliable and repeatable information of the AP in immuno detection systems. It is possible that other commercial antiserum to human (or mouse) AP could be used to assess bovine AP by

Western blot, however each antiserum would need to be assessed for cross reaction and specificity for bovine AP confirmed before use.

The discovery of high AP activities in the bovine nasal mucosa suggests an important physiological role for this enzyme. Although to date there has been several biochemical evidences on AP function scientifically reported, the most well-researched AP function has been in its major involvement in bone mineralization (Yoon et al. 1989; Beertsen and van den Bos 1992 and Fedde et al. 1999) and dephosphorylation of adenosine nucleotides (Gallo et al., 1997).

Fractionation of AP into its isoenzymes is useful in distinguishing its molecular characteristic and differentiating nasal AP from AP in other tissue. Preliminary study was first performed by separating AP from NS and other tissue extract with SDS-PAGE and stained with 5-Bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium chloride (BCIP/NBT) substrate and the gel was observed every hour for up to 5 h at 37 °C. However, no bands were observed to be present in NS or any of the tissue extracts following SDS-PAGE. This may be due to the presence of SDS in the polyacrylamide gel, denaturing and running buffers where SDS exhibited a strong inhibitory effect on AP activity (Bortolato et al., 1999 and Zappa et al., 2001). Furthermore, it was suggested that the heating process at 95 ºC for 4 min involved during sample preparation could inactivates the AP activity (Ikehara et al., 1978 and Yasuura et al., 1985). However there were still no visible bands present on the polyacrylamide gels run in the absence of SDS and without a reduction step.

Attempting to devise an alternative electrophoretic method, agarose electrophoresis was performed. With agarose, the migration of non-reduced large protein or complexes occurs as is likely to be the reason for detecting AP after the electrophoresis. Agarose gel has been able to show the electrophoretic mobility for all the samples except from heart sample. Apart from the band produced from liver extract which shows the most anodal from the origin, the intestine and kidney extract produced bands having lower electrophoretic mobility than the NS and nasal mucosa extracts though the variation seen could not be distinctly visualized. The separation produced was not distinct and samples from the NS, nasal mucosa and bone had smeared appearances. These smearing seen may be avoided by increasing the dilution of samples loaded in the agarose gels,

Isoelectric focusing was most effective of the electrophoretic methods in relation to characterization of the enzyme and the separation of high-specific activity preparations. In this study, IEF demonstrated differences between the AP of NS and

extracts of AP from other tissues. The AP in NS and in the mucosa had pIs of 5.2 and as such had a different pattern of AP isoforms compared to AP from other tissues. These differences are either resulting from the expression of a different gene in the mucosa or from the same gene product being changed by PTMs. An investigation of the gene responsible in the nasal mucosa for production of AP in NS is necessary before this question can be settled. Furthermore the discovery of AP in nasal epithelium and NS in cattle presents opportunities for further investigation of the role of the enzyme in the susceptibility of mammals to infection through the nasal route.

The AP have been postulated to be involved in a range of other processes, including cell adhesion (Hui et al. 1993), vitamin B transport (Rindi et al. 1995), metastasis (Manara et al. 2000), and cell signaling (Swarup et al. 1981; Muller et al. 1991). Recent studies of the biological function of AP have revealed that the enzyme has the ability to dephosphorylate LPS endotoxin from Gram-negative bacteria (Poelstra et al., 1997; Koyoma et al., 2002 and Beumer et al., 2003) reducing the toxic effects of LPS (Bates et al., 2007) and, in the intestine, can be induced by Resolvin E1, an anti-inflammatory derivative of omega-3 fatty acids (Campbell et al., 2010). Thus intestinal AP has a major function in reducing the effects of LPS from intestinal bacterial flora (Lalles, 2014) and may have a similar function in livestock (Mani et al., 2012).

If AP in NS has the same activity on LPS then its presence in this secretion could contribute to the host defences by acting against invading pathogens in the respiratory tract responsible for BRD. It is of interest that AP in bronchial and alveolar fluid is believed to function in the extracellular dephosphorylation of ATP to fuel the activity of cilia in the airways (Picher, 2003) while there may also be an interaction between ATP and nasal AP in minimizing inflammation as has been suggested for IAP (Lalles, 2014). Furthermore in this species, NS can be transferred to the mouth as cattle have the tendency to lick their muzzle and nostrils so that the nasal AP could be effective in the rumen as an anti-endotoxin with a dephosphorylation activity (Lalles, 2014).

In conclusion, this chapter has demonstrated that NSAP was produced locally and secreted by the nasal epithelial cells. This study also illustrates characteristic molecular differences between the isoenzymes of NSAP as demonstrated by IEF methodology. The bovine NS and nasal mucosa isoenzymes have been characterized with respect to their pIs and compared them with other bovine tissues known to produce AP. Whether nasal AP isoenzymes represent specific biomarkers for the response of local innate response, in particular in the ability to dephosphorylate LPS, or merely reflect intermediate products of nasal AP biosynthesis remains to be

determined. In order to fully understand the molecular differences that define the bovine nasal AP isoenzymes and their clinical significance, subsequent work will be described in the next chapter which includes a detailed genomic characterization of the AP gene expression.

Chapter 4

Expression of nasal-associated alkaline phosphatase in bovine nasal tissue

4.1 Introduction

As detailed in Chapters 2 and 3 AP activity in NS has been shown to be high and it was demonstrated by histochemistry and tissue extraction that the NSAP is present in the nasal mucosa. Results from the IEF have also shown that NSAP and AP from nasal mucosa are the same isoform, having the same pI value but different from other AP related tissues suggesting that the NSAP is a different isoenzyme from the AP from other tissues, but it could still be that it is the same gene product but with different post translational modifications. In order to determine if the AP is produced locally and to characterize the nature of the isoenzyme, it was necessary to confirm the expression and determine the sequence of an AP gene in the nasal mucosa and to determine if the difference from other APs is at the gene level.

In bovine, previously characterised APs are encoded by multiple genes that can be classified by their expression patterns into two general groups, the tissue-non-specific APs also known as *ALPL* for their liver expression and the tissue-specific AP known as *ALPI* due to being expressed primarily in the intestine. The physiological role of tissue non-specific AP as a phospho-monoesterase with a high optimum pH, which is not found in mammalian tissue, has yet to be defined in most tissues although there are numerous suggestions (McComb et al., 1979). This is especially true for the nasal AP which has been identified by this research. One of the better studied biological functions of APs is the role of bovine *ALPL* in osteogenesis by promoting bone mineralization, as demonstrated by the presence of collagenous and non-collagenous proteins due to mutation that results to *ALPL* deficiency in cattle (Fisher et al., 1987). However a relatively recently and physiologically relevant AP action that has been identified, is the detoxification of the LPS by bovine IAP (Goldberg et al., 2008 and Lalles, 2010). The *ALPI* has been implicated in mediating host-bacterial interactions through its ability to dephosphorylate lipid A of the Gram-negative bacterial cell wall component LPS (Lalles, 2010).

As the name suggests non-specific AP gene is expressed in various tissues mainly liver, kidney and bone. Histochemical distribution of AP within the bovine liver is mainly present in the bile canaliculi as compared to human where the strongest reaction is seen in the sinusoids and the endothelium of the central and periportal veins (Ordy et al., 1966). In kidney, AP is mainly distributed at the brush border membrane of the renal proximal convoluted tubule (Yamamoto and Yasuda, 1973 and Prasad et al., 2005). The presence of AP in bone has long been described originally by Bourne (1943) and Pritchard (1952), showing the source of bone AP is found mainly in osteoblast and

predominantly located in the plasma membrane (Gothlin and Ericsson, 1971). In mammals, *ALPI* is expressed by intestinal epithelial cells and is enriched in vesicles that are actively released from the cell microvillar tips into the intestinal lumen (McConnell et al., 2009; Shifrin et al., 2012).

Yang et al. (2012) reported that all mammals including cattle had a single AP gene at the *ALPL*-like locus, which was surrounded by genes that were orthologs in all species with locally well-assembled genomes, showing that this region of the genome has been well-conserved among mammals. However, the genomic situation at the *ALPI*-like locus differed greatly among taxa, with several species having multiple *ALPI*-related genes. Since the discovery of the ability of bovine IAP to dephosphorylate LPS, various genomic investigations have identified 6 *ALPI*-related genes named *ALPI*-like1-6 (Yang et al., 2012) but only four of which (*ALPIl2*; E1BJE3, *ALPIl4*; ENSBTAT000002229, *ALPIl5*; F1N2M5, and *ALPIl6*; F1N6T5) are annotated in Ensemble.

Alkaline phosphatase represents a family of conserved isoenzyme identified in many animal species. In bovine, AP exists as several isoenzymes in different tissues. Within the respiratory tissues, non-specific AP activity has been previously demonstrated within the cell and on the surface of the lower respiratory epithelium in humans, where it is able to dephosphorylate ATP to AMP and adenosine, important for mucociliary clearance (Picher et al., 2003). The enzyme was also reported several decades ago in human nasal mucosa (Bourne, 1948) and more recently described in this tissue though only in micro litre volumes of NS of guinea pigs (Gawin et al., 1991) and in the related olfactory epithelium of mice and rats (Gladysheva, 2002). Heat stability analysis on human serum AP has been used as a means of identifying the organ origin of the enzyme contributing to the total enzyme activity in the serum (Tan et al., 1972). Bone AP was found to be more heat-labile than liver AP and the two can be differentiated by pre-incubating the serum at 56° for 10 min before enzyme assay (Tan et al., 1972 and Anderson et al., 1990). The expression level of heat stable AP using histochemistry for the detection of pneumocyte type-1cell was reported to be 10 fold lower in human in compare to monkeys (Nouwen et al., 1990). Up to the present time there have been no studies to determine whether AP gene expression is active in the nasal mucosa of human or other animal.

4.1.1 Coding for gene expression

Reverse transcription polymerase chain reaction (RT-PCR) represents a powerful indirect method to analyse RNA levels (Freeman et al., 1999 and Erlich, 1999) and gene

characterisation by standard sequencing of the PCR product. Sequence analysis of PCR product would then compare with the AP gene to confirm any genetic divergence. In eukaryotes most of the gene protein coding region is not a continuous DNA sequence reading out co-linearly into messenger RNA (*mRNA*) but is composed of an alternating array of coding sequence, called exons separated by intervening pieces of non-protein coding DNA referred to as introns. The removal of these intronic sequences from the initial RNA transcript and the joining of all exons in a head to tail arrangement occur, through a precise mechanism mediated by the splicing machinery in the nucleus (Weiner, 1993), to form a *mRNA* molecule. Genomic DNA containing introns can be used to express the gene of interest but these sequences can span up to hundreds of kilo bases and are therefore impossible to clone into the currently available mammalian expression vectors. Alternatively *cDNA*, which is a direct copy of the *mRNA* transcript, can be made *in vitro*, and used to directly transcribe and translate genes to a functional protein. Thus, the *cDNAs* encoding the bovine genes *ALPL* and *ALPI* have been used in this chapter.

4.1.2 Aims of Study

The aims of the work presented in this chapter were to:

1. Investigate the expression of the AP gene in bovine nasal mucosa

2. Exploit the difference in coding sequences of AP isoform to design primers to differentiate between the bovine *ALPL* and *ALPI* genes

3. Determine the sequence conservation/similarity between bovine tissue non-specific AP and IAP with nasal derived AP

4.2 Materials and methods

4.2.1 Sample material

Nasal mucosa, liver and small intestine tissue were collected from the same cattle (n=6) used for the preparation of tissue extracts (as described in section 3.2.1.1) within 1 hr of euthanasia. Tissue sections (1 by 1cm square and 1cm thick) were collected and preserved in RNA*later* (Ambion, Darmstadt, Germany). The samples were stored at -80°C until RNA extraction.

4.2.2 Total cellular RNA extraction

Approximately 100mg of tissue samples were disrupted using a blade and was placed into a RNase-free microcentrifuge tube for RNA preparation using PureZOL™ (Bio-Rad Laboratories, Hemel Hempstead, UK) according the manufacturer's protocol. The lysate was homogenised by repeated passage through a clean syringe and 23-gauge needle (10 times). The homogenised lysate was centrifuge at 10,000 x *g* for three min at 20°C.

RNA was precipitated from the tissue lysate supernatant with 70% (v/v) ethanol and applied to RNeasy® mini column (Qiagen, Crawley, UK) and centrifuged at 8000 x *g* for 15 sec. The flow through was discarded and the column was placed into a new tube. The RNeasy® mini column was washed by adding 350 µl RW1 buffer (washing buffer consists of guanidine salt and ethanol) and centrifuged at 8000 x *g* for 15 sec. Possible genomic DNA contamination was eliminated by including DNase treatments. DNase incubation mixture was added onto the RNeasy® mini column and incubated for 15 min and spun at 8000 x *g* for 15 sec and washed with 350 µl RW1 buffer and centrifuged at 8000 x *g* for 15 sec. The column was washed twice by adding 500 µl of RPE buffer and centrifuged at 10,000 x *g* for two min. To elute the RNA, the column was transferred to a new RNase-free microcentrifuge tube. Thirty µl of RNase-free water was added directly onto the centre of the silica gel membrane and centrifuged at 10,000 x *g* for one min. All steps were carried out at 20 to 25°C. The RNA samples were stored at -80°C until further analysis.

4.2.3 Estimation of the quantity and integrity of total RNA

The isolated RNA was spectophotometrically quantified by using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Epsom, UK) at absorption of 260 nm. The RNA

purity was assessed by measuring the ratio of the absorbance at 260 and 280 nm. A typical ratio of 1.8 to 2.0 was obtained for most of the samples which is acceptable.

4.2.4 *cDNA* synthesis

Reverse transcription (RT) reactions were performed with RNA samples from nasal mucosa, liver or small intestines. Two µg RNA samples were denatured at 65°C for 5 min. Fifty µl of the reaction mix consisting of 2µg of total RNA sample, 1x First strand buffer, 0.5mM each of dNTPs, 10mM dithiothreitol, 3µg random primers, 20U of RNAseOut and 400U M-MVL reverse transcriptase (Life Technologies Ltd, Paisley, UK) were incubated for 30 min at 37°C, 60 min at 42°C, 15 min at 70°C and then held at 4°C.

4.2.5 Analysis of total RNA

The mRNA of nasal mucosa and other tissue AP were analysed on 2% (w/v) agarose gels containing ethidium bromide in 1 X TAE buffer and ran at 80 V for 20 min to assess the integrity of the total mRNA.

4.2.6 Primer design

Coding sequences (CDS) of the bovine IAP gene *APLI* (NM_173987) and non-specific AP gene *ALPL* (NM_176858) were derived from the National Centre for Biotechnology Information (NCBI) website. Primers were designed using an interactive web-based primer program algorithm, GeneFisher software version 1.2.2 (BiBiServe, Bielefeld, Germany), the primers were synthesised commercially (Eurofins, Germany). The primer details are shown in Table 4.1.

4.2.7 Polymerase chain reaction

A total of volume of 25µl PCR reaction mix comprising 12.5µl RedTaq® DNA polymerase buffer (Sigma Chem. Co, Poole, UK), 0.5µl of each primer (5pmol per reaction tube), and 200ng gDNA. Amplification conditions were 30 cycles (94°C for five min, 94°C for one min, 58°C for one min, 72°C for one min, 72°C for 10 min). The PCR products (2µl) were visualised using 0.5ug/ml ethidium bromide stained agarose gel and quantified against a 100bp DNA mass ladder (Life Technologies, Paisley, UK).

4.2.8 Purification and sequence analysis

Polymerase chain reaction products were purified using QIAquick PCR purification kit (Qiagen, Crawley, UK) according to manufacturer's instructions. The PCR products were sequenced using Big Dye v3.1 Terminator (Life Technologies, Paisley, UK) in 10µl reaction volume containing 200-500ng template, 1X sequencing buffer, 32mM primer and 1x Big Dye mix.

The sequencing reactions were carried out in two randomly chosen nasal mucosa samples with forward and reverse primers in separate reactions. The sequencing products were ethanol precipitated, resuspended in formamide and read using capillary electrophoresis on Applied Biosystems 3130XL Genetic Analyzer (Hitachi, Tokyo, Japan). The 'BLAST' search engine within the NCBI database was used to analyse sequence data (Figure 4.1).

4.2.9 Genomic analyses

AP related sequences were obtained from sequenced genomes present in NCBI and Ensembl by BLAST searches using the bovine *ALPL* (NM_176858) and *ALPI* (NM_173987) sequences as query. Primer sequences were retrieved and subjected to alignment and phylogenetic analysis using UniProt software from EMBL-EBI.

The BLAST search engine within the NCBI database (www.ncbi.nih.gov/BLAST/) was used to analyse sequence data.

	Accession number	Primer notation	Sequence 5′-3′	Sequence coordinates	Product length
ALPL	NM_176858	*ALPL*-F	GACAGCTGCCCGCATCCTCA	489-989	500
		ALPL-R	CCTTCTCCATCCAGCTCATACTCCA		
ALPI	NM_173987	*ALPI*-F	GGGAGTGGTGACCACCTCCA	206-706	500
		ALPI-R	GTCAATGCGGCCTCCCTCCA		

Table 4.1: Primer sequences generated and used in RT-PCR for the amplification of bovine AP present in nasal, liver and intestinal tissues.

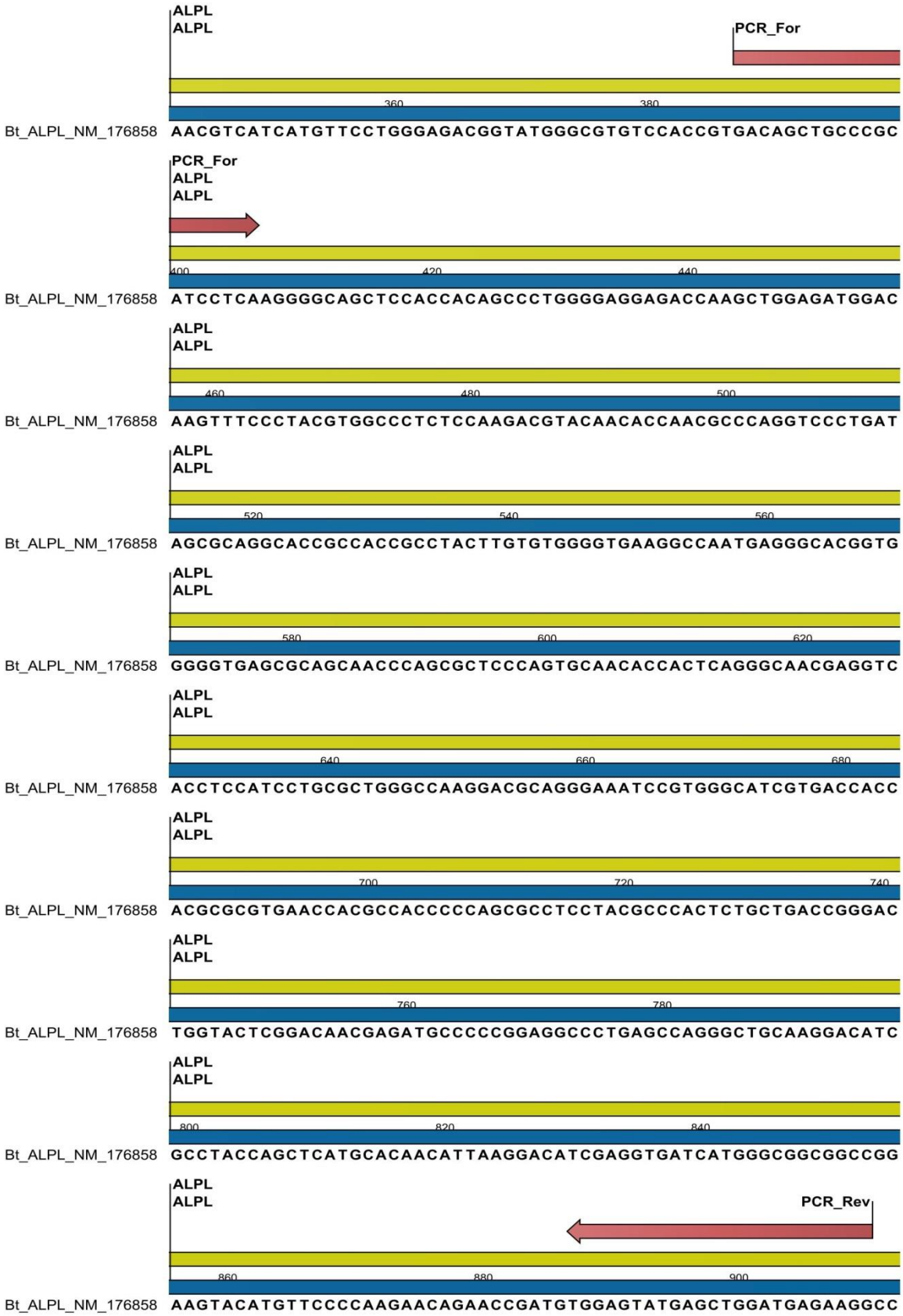


Figure 4.1: The nucleotide sequence of the *ALPL* gene fragment (NM_176858) amplified by RT-PCR.
Yellow=AP coding sequence; Blue=ALPL gene sequence and Red=forward and reverse primers.

4.3 Results

4.3.1 Analysis of RNA quantity and quality

Full-length total RNA was isolated from the bovine nasal mucosa tissue of 6 dairy cows. Good quality RNA samples were obtained as determined by ultraviolet spectrophotometry (A260/A280 value > 1.8) and agarose gel electrophoresis assessment (Figure 4.2); revealing intact 18s and 28s ribosomal RNA species.

4.3.2 PCR amplification and analysis of nasal mucosa AP mRNA

Bovine nasal mucosa AP *cDNA* amplified by RT-PCR produced a single PCR product at the predicted size of 500bp with primers against specific regions for bovine *ALPL* gene (Figure 4.3a). Alkaline phosphatase primers derived from *ALPL* also recognises cDNA for liver therefore suggesting that the nasal mucosa AP having the same gene with liver AP.

However, there was no amplification of nasal mucosa *cDNA* with primers for the bovine *ALPI* gene (Figure 4.3 b). Alkaline phosphatase primers derived from *ALPI* only recognises cDNA for intestinal tissue. The used of liver and intestine *cDNA*s also served as positive and negative controls for each primer pair used in this experiment.

4.3.3 Sequencing of PCR product

The nucleotide sequences of purified PCR products were determined using genetic analyser and assessed with CLC Genomic Workbench Version 5.5.2 (CLC bio, Cambridge, UK). The nucleotide sequence was compared to the NCBI database to identify those genes which shared the greatest sequence identity with bovine nasal tissue sample. Sequences of the PCR products from two biological samples of nasal tissue amplified with *ALPL* primers, showed complete homology with this region of the *ALPL* splice variant (Figure 4.4).

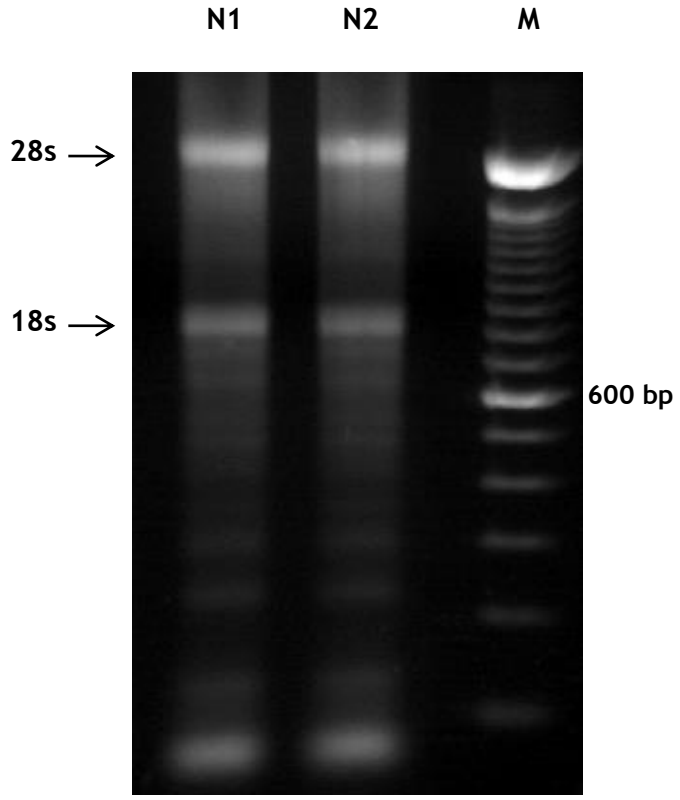


Figure 4.2: Ethidium bromide stained agarose gel showing the integrity of the 18s and 28s ribosomal RNA components.
N1=nasal mucosa 1; N2=nasal mucosa 2 and M=Marker (100 bp DNA ladder).

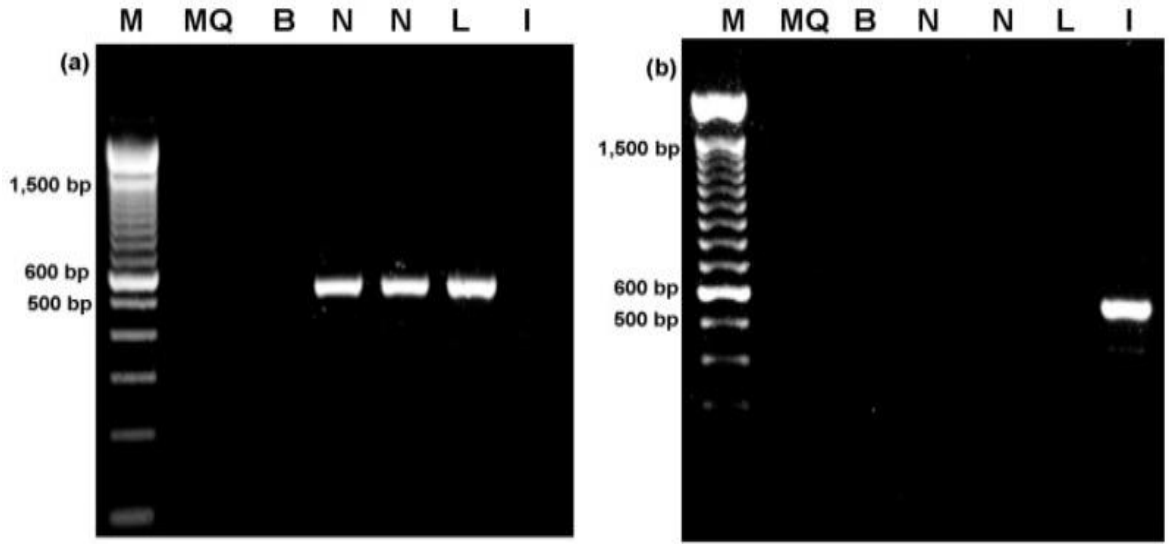


Figure 4.3: Alkaline phosphatase mRNA analysis from various bovine tissues by RT-PCR. The presence of a single 500 bp DNA fragment corresponding to the predicted product size was visualised when using (a) tissue non-specific AP primers (*ALPL* gene) and (b) intestinal AP primers *ALPI* gene. PCR products were present in nasal mucosa only when non-specific AP primers were used. M=Marker (100 bp DNA ladder); MQ=high purity water; B=blank; N=nasal mucosa; L=liver; I=intestine.

Figure 4.4: The nucleotide sequence of bovine nasal mucosa mRNA shows 100% homology to the bovine *ALPL* gene (NM_176858). Box in red are the forward and reverse primers used for amplification by RT-PCR.

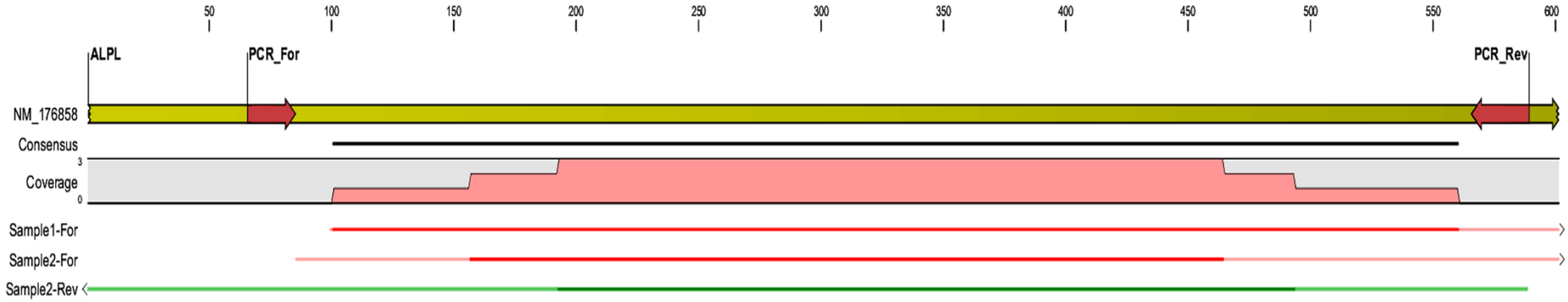


Figure 4.5: Gene sequence of PCR product from 2 different samples of AP from bovine nasal mucosa shows 100% homology with bovine *ALPL* gene.

4.4 Discussion

The findings from the histochemistry study (Chapter 3) demonstrated the presence of AP in the nasal mucosa and the evidence of strong AP activity presence along the epithelium and mucosal glands suggesting that the AP was produced locally. In order to substantiate this finding, RT-PCR analysis was performed on RNA extracted from the bovine nasal mucosa from four healthy cows. The data obtained showed that a clear PCR product at 500 bp was observed when primers derived from the *ALPL* gene were amplified. However, there is no product detectable when the primers corresponding to the bovine *ALPI* gene were used in the RT-PCR reaction, thus indicating that only the tissue non-specific AP is produced and expressed locally in the bovine nasal mucosa.

While the liver has long believe to be the major site for tissue non-specific AP production, this enzyme has been considered to be present and expressed also in the mammalian kidney, bone and placenta (Goldstein et al., 1980; Hahnel et al., 1990; Ali et al., 1996; Prasad et al., 2005 and Zimin et al., 2009). The tissue non-specific AP has also been reported to be present in brain tissue (Brun-Heath et al., 2011) and circulating B cells in human (Hossain and Jung, 2008). The assay of AP in serum has been used as a marker of liver function for several decades (McComb et al., 1979) though bone AP can contribute to the serum activity. The tissue non-specific AP and IAP genes are different, reflected in their different patterns of tissue expression. The ubiquitously expressed *ALPL* plays an important function in bone mineralization in bovine (Fisher et al., 1987). In humans, over 261 different mutations in *ALPL* have been linked to hypophosphatasia and skeletal abnormalities, of which 75% are missense mutations. Several of these mutations result in dominant inheritance of the disease (Mornet et al., 2011), suggesting that modest changes in AP function at sites of bone mineralization can lead to deleterious phenotypes, by restricting the evolution of copy number variation of this gene once it became dedicated to this function.

Although the principal function of NSAP remains in doubt, the histochemistry results in Chapter 2 have demonstrated that strong AP activity was present at the epithelia of the nasal mucosa and further investigation could be done to determine whether nasal AP bio-synthesis occurs in the cytoplasm which is localized exclusively in the cytoplasmic membrane (periplasm) or the outer membrane as seen in most prokaryotic cells (Hoffman and Wright, 1985; Klionsky and Emr, 1989; Matsumoto et al., 1990 and Qureshi et al., 2010). Alkaline phosphatase expression in the goblet cells, entero-endocrine cells, and absorptive epithelial cells (brush borders) of the intestines expressing AP (Mutoh et al., 2002), as well as in the uterine body in human (Franco et al., 2012) may

reflect a role in surface epithelial protection for this enzyme. Recent studies of the biological function of IAP have revealed that the enzyme has the ability to dephosphorylate LPS endotoxin from Gram-negative bacteria (Poelstra et al., 1997b; Koyama et al., 2002 and Beumer et al., 2003) to reduce the toxic effects of LPS (Bates et al., 2007). Bovine IAP has a major function in reducing the effects of LPS from intestinal bacterial flora (Lalles, 2010 and Campbell et al., 2011). Possibly a similar function occurs for NSAP but will need further investigation to verify.

The specific reason as to why nasal tissues expressing *mRNA* encoding the tissue non-specific AP genes is unclear, but the induced expression probably results from the reaction of certain cytokines or hormones with their cellular receptors in response to invading pathogens and inflammatory signals. We have known that AP in NS is produced in the nasal mucosa and if AP in NS has the same activity on LPS then its presence in this secretion would contribute to the host defences by acting against invading pathogens. It is of interest that AP in bronchial and alveolar fluid is believed to function in the dephosphorylation of ATP to fuel the activity of cilia in the airways (Picher et al., 2003).

Additional studies will be necessary to understand the role of AP present in the bovine nasal mucosa and NS. However, based on the sequence similarity/conservation of nasal AP with tissue non-specific AP it is possible that it may play a role in dephosphorylation of LPS, modulating immunological responses that are essential to protect the host against infection and restore homeostasis. The expression of nasal AP on surface epithelia of the nasal mucosa as demonstrated in Chapter 3 could indicate a protective role for the enzyme, situated in a position to communicate with the external environment and protect local tissues from invading pathogens such as LPS producing bacteria.

Since both of the AP genes encode enzymes with well conserved catalytic activities, it strongly suggests that NSAP may has the same capability to dephosphorylate LPS. The assumption is based on the same physiological requirement and environmental condition where IAP is produced. Similar to the intestine, the nasal mucosa is also a major site of infection, being where the onset of respiratory diseases usually happens. Gram-negative bacteria like *M. haemolytica* and *P. multocida* are commonly present in the nasal mucosa and capable of causing pneumonia in cattle (Whiteley et al., 1992 and Senthilkumaran et al., 2013). Therefore, the presence of high AP activity and tissue AP expression in the nasal mucosa could mirror the capacity of IAP to detoxify LPS and may be important for modulating innate immune responses to LPS producing bacteria. It

could be hypothesized that the infection with Gram-negative bacteria causing BRD in cattle may have been the driving force that lead to the presence of nasal AP being secreted by the nasal tissues and shaped the rapid evolution of the *ALPL* gene expression.

The results of this chapter confirm that the presence of AP in NS is due to local production in the bovine nasal mucosa with the expression of a local *ALPL* gene. Furthermore there is the possibility that AP activity is up-regulated during infection or inflammation of the upper respiratory system. Hence, further work is needed to investigate this finding. Microarray technology could be used to monitoring the changes of AP gene expression during healthy and diseased phase in parallel. *In-situ* hybridisation could be performed to give a more detailed analysis and specific localization of AP mRNA within the respiratory cells of nasal mucosa.

Chapter 5

Proteomic investigation of bovine nasal secretion

Chapter 5

5.1 Introduction

Determination of potential biomarkers in bovine NS of healthy and diseased animals might contribute to the early diagnosis of respiratory diseases in cattle. Nasal secretion contains a variety of proteins derived from plasma, cellular and glandular sources from both mucous cell and serous cell secretions such as lysozymes, lactoferrin and immunoglobulins (Baraniuk, 2000; Baraniuk and Staevska, 2004). As in humans, bovine NS contains similar proteins that are capable of forming a barrier against microbial infections transmitted through aerosols. Determination of the normal profile of bovine NS is essential to determine the physiological function of the proteins present in NS and identify disease-specific changes. Therefore, the understanding of the proteome map and identification of proteins in bovine NS could provide an important insight into the innate and acquired immune defence mechanism involve in the development of respiratory diseases. Passalacqua et al. (2001) demonstrated that there is a similarity in mucosal response patterns between the upper and lower respiratory airways hence any potential biomarkers identified at the nasal mucosa could provide a similar view on the immunological responses happening in the lungs.

The nasal mucosa is designed to regulate airflow and permit exudation of interstitial fluid to provide water for humidification and ciliary activity. The nasal mucosa glands contain serous and mucus cells. Generally, the major protein classes detected in human NS reported by Casado et al. (2005) and Baraniuk et al. (2000) are cytoskeletal proteins (33%), innate (27%) and acquired immunity (21%) system proteins and other cellular proteins (20%). Host defence is a prominent function of NS. Among the components of the secretions, the mediators of adaptive immunity include IgA and IgG, which are produced by plasma cells juxtaposed to submucosal glands. The immunoglobulins were also believed to act in the lumen of the airway and on the mucosa to prevent the attachment and invasion of pathogenic organisms. The cytoskeletal proteins may have been derived from the turnover of epithelial lining manipulation. Mediators of innate mucosal host defence are also found in NS and include substances that selectively disrupt bacterial cell walls and membranes, requisition microbial nutrients, or act as traps for microbial attachment.

Proteomics is the study of the entire proteins and peptides in an organism or a part of it, such as a cell (Peddinti et al., 2010), tissue (D'Ambrosio et al.,2005 and Beddek et al., 2008), or fluids like NS (Casado et al., 2005), bronchoalveolar lavage fluid (BALF) (Racine et al., 2011), plasma (Henning et al., 2014), serum (Alonso-Fauste et al., 2012), uterine fluid (Faulkner et al., 2012), urine (Pyo et al., 2003), or saliva (Ang et al., 2011

and Lamy et al., 2012). It is widely believed that changes in the composition of the proteome could mirror disease states and provide evidence to its origin, eventually leading to targets for early diagnosis or new treatments. Proteome analysis presents a major task due to its complexity, however MS has proven to be a central analytical technique for tackling this kind of studies (Tanaka et al., 1988 and Link et al., 1999). An important advantage of using proteomic is the ability of this method to provide an impartial approach which allows the investigation of numerous potential biomarkers that may carry specific information of a disease. Lindahl et al. (1998, 1999, 2001 and 2004) has demonstrated a comprehensive investigation of proteins of human broncheoalveolar lavage and NS which found many potential biomarkers in asthmatic and allergic rhinitis patients such as the involvements of an altered form of lipocortin-1 and Clara cell protein-16 in BALF and NS of smokers. Those investigations were performed using 2-DE as the separation method and identification using MALDI-TOF-MS.

The key advantage of applying proteomic methods in the study of NS is its ability to provide an unbiased approach that allows the investigation of numerous putative biomarkers that could represent the health status or respiratory disease information of the animal. Laemmli (1970) reported on the application of SDS-PAGE and later in 1975, O'Farrell demonstrated the usage of 2-DE by including isoelectric focusing as the first stage. These two techniques basically separate proteins according to two intrinsic characteristics: pI and molecular weight.

Separation is accomplished in two steps; proteins are first fractionated by isoelectric focusing (proteins migrate to their pI in a pH gradient) and then in a perpendicular dimension by SDS-PAGE. A characteristic protein spots is obtained from any sample analysed. After the 2-DE separation, the difficulty to identify these protein spots remains. These gel separation techniques have been successfully used for more than 30 years and are still widely used (Zürbig and Jahn, 2012). A major advance in the identification of proteins was achieved with the implementation of MS. The first step in this process of identifying proteins in the spots (2-DE) or bands (1-DE) by MS is to subject them to a proteolytic, usually trypsin in-gel digestion (Henzel et al., 1993 and Henzel et al., 2003).

5.1.1 Aims of study

The aims of the work presented in this chapter were to:

1. Identify the proteins present in healthy NS from individual animal using 1-DE
2. Investigate variation of protein spot intensity in NS across healthy animals
3. Identify the proteins present in 2-DE from pooled healthy NS samples

5.2 Materials and methods

5.2.1 Bovine nasal secretion samples

Eight bovine NS samples for 1-DE and 6 bovine NS samples for 2-DE from the same group of animals described in section 2.2.1 were randomly selected and used in this study. The NS was collected as described in section 2.2.2.

5.2.2 Protein quantification

The protein concentration of the pooled samples was determined by Bradford assayas described in section 2.2.3.

5.2.3 One-dimensional gel electrophoresis

Ten-µg of protein from each NS was utilised for this experiment and 1-DE was carried out on the Criterion electrophoresis system from Bio-Rad according to steps described in section 2.2.8. Nasal secretion sample at 1/20 dilution were loaded in well 1-10 with approximate concentration of protein loaded correspond to Table 2.4.

5.2.4 Sample preparation for two-dimensional gel electrophoresis

Five µl of NS sample from 6 different healthy animals were used for 2-DE separation. These were carried out individually on 2-DE gels to observe any variation of protein separation between the samples. Protein concentrations were determined to allow dilution of samples to an equal protein loading of 200 µg for 2-DE protein separation first by its pI (IEF) and later by its Mw (SDS-PAGE).

Subsequently, 5 µl of NS sample from each of the 6 healthy animals, used in the previous investigation were pooled for 2-DE separation. Pooled samples were run on duplicate gels and major protein spots selected and analysed by MS for protein identification.

5.2.5 Two-dimensional gel electrophoresis

Separation by pI was carried out using 11cm immobilized pH Gradient (IPG) strip with a pH range of 3 to 10 (Bio-Rad, Hemel Hempstead, UK). After 200 µg of protein loading on the IPG strips, with NS diluted in a rehydration buffer (8M Urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte®) (Bio-Rad, Hemel Hempstead, UK) and covered in 500µl of mineral oil, a

combination of rehydration and focusing step was carried out over 17 hr with a total of 35000 V-hr. The IPG strips were then removed, oil drained with filter paper and then treated with two equilibration buffers both made from a stock solution comprised of 6M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, the first of these containing 2% (w/v) dithiothreitol (Sigma-Aldrich, Poole, UK) to reduce the proteins and subsequently the alkylating agent iodoacetamide at 2.5% (w/v) (Sigma-Aldrich, Poole, UK) both were incubated for a duration of 15 min each. IPG strips were then placed onto Criterion™ XT Bis-Tris Gel and submerged in Tris-glycine running buffer on a Criterion™ electrophoresis system at 200 volts for 1 hr (Bio-Rad, Hemel Hempstead UK).

5.2.6 Gel staining and image acquisition

Polyacrylamide gels were stained and the gel images were acquired accordance to the steps described in section 2.2.8.4.

5.2.7 Statistical analysis of variation on protein spots expression

Two-DE gel images were uploaded onto Nonlinear Progenesis SameSpots 2D gel image analysis software (Nonlinear Dynamics, Newcastle, UK) which was used to identify protein spots which were differentially expressed between 6 individual NS samples (inferred by the programme by normalised spot intensities). Initial results were filtered using the programme's statistical analysis function, with only those with a power value of >80% and ANOVA significance score of <0.05 between groups of individual gels, being chosen for protein identification.

5.2.8 Tryptic in-gel digestion of proteins

Protein spots were excised using a scalpel blade from the 2D gels of the pooled NS samples, diced if necessary and placed into eppendorf tubes and washed in 500µl of 100mM ammonium bicarbonate for 30 min. This wash was poured away and the gel pieces washed twice in 500µl of 50% acetonitrile/100mM ammonium bicarbonate for 30 min to de-stain the gel pieces. The gel pieces were finally washed in 50µl of acetonitrile to shrink the gel pieces for 10 min, after which the solvent was removed and the gel pieces air dried completely in a vacuum centrifuge for 2 hr. In gel digestion was performed with sequencing grade trypsin (Promega, Southampton UK, V511A) which was re-suspended in 20µl of 25mM ammonium bicarbonate and added to the dried gel pieces. Once the trypsin has absorbed and the gel pieces fully rehydrated a further 20µl of 25mM ammonium bicarbonate was added over the gel pieces.

The protein was allowed to digest overnight at 37°C. The following day the Eppendorf tubes were pulse centrifuged to pellet the gel pieces and the liquid transferred to a 96 well plate. To the remaining gel pieces, 20µl of 5% formic acid was added and allowed to incubate for 20 min on the shaker after which 40µl of acetonitrile was added and the incubation continued for a further 20 min. The gel pieces were pelleted and the liquid added to the 96 well. The combined extracts on the plate were dried down in a vacuum centrifuge.

5.2.9 Mass spectrometry analysis

Peptides were solubilized in 0.5 % formic acid and fractionated on a nanoflow uHPLC system (Thermo RSLCnano) before online analysis by electrospray ionisation (ESI) mass spectrometry (MS) on an Amazon ion trap MS/MS (Bruker Daltonics). Peptide separation was performed on a Pepmap C18 reversed phase column (LC Packings), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min at a flow rate of 0.2 µl/min. Mass spectrometric analysis was performed using a continuous duty cycle of survey MS scan followed by up to ten MS/MS analyses of the most abundant peptides, choosing the most intense multiply charged ions with dynamic exclusion for 120s (Burchmore, pers. comm. 2013).

5.2.10 Protein identification

Mass spectrometry data was processed using Data Analysis software (Bruker) and the automated Matrix Science Mascot Daemon server (v2.1.06). Protein identifications were assigned using the Mascot search engine to interrogate mammalian protein sequences in the NCBI Genbank database for Bos taurus, allowing a mass tolerance of 0.4 Da for both MS and MS/MS analyses (Burchmore, pers. comm. 2013). Analysis of the MS and MS/MS spectral data was performed using SwissProt database version 57.15. Protein scores with CI.95% were considered indicative of a positive identification with individual ion scores at P value 0.05 or better, indicative of identity or extensive homology.

Tryptic digestion, ESI-MS/MS and protein identification were performed in Glasgow Polyomics with the MS analysis and initial Mascot search being undertaken by Ms. S. Eadie.

5.3 Results

5.3.1 One-DE on individual nasal secretion samples

Preliminary observation of 1-DE on bovine NS shows no distinctive difference in term of protein band variation between 10 individual NS samples. A total of 13 distinctive bands within a track (Figure 5.1) were excised and processed for protein identification. Upon tryptic digestion and MS, the 11 proteins with highest MOWSE scores in each band are identified in Table 5.1.

As predicted in Chapter 2, band f and g were identified as albumin and Ig heavy chain respectively. Immunoglobulin light chain was found near to the predicted band size at band j below apolipoprotein A1 which have a molecular weight of around 30 kDa. Protein having anti-microbial properties such as lactoferrin, serotransferrin and cathelicidin were also identified. Odorant binding protein, low-Mw soluble proteins highly concentrated in the nasal mucus of vertebrates which is involved mainly in scent recognition and associated with pheromone-sensitive neurons was identified at band k. Other protein bands that were identified were alpha-2-macroglobulin and bovine cytoskeletal keratin derived from the nasal epithelium during sampling of NS.

In addition, Table 5.2 tabulated by name and NCBI accession number all the protein hits identified in respective of the protein bands from 1-DE separation that have MOWSE score of >100 and peptide matches of >3 (UniProt Reference).

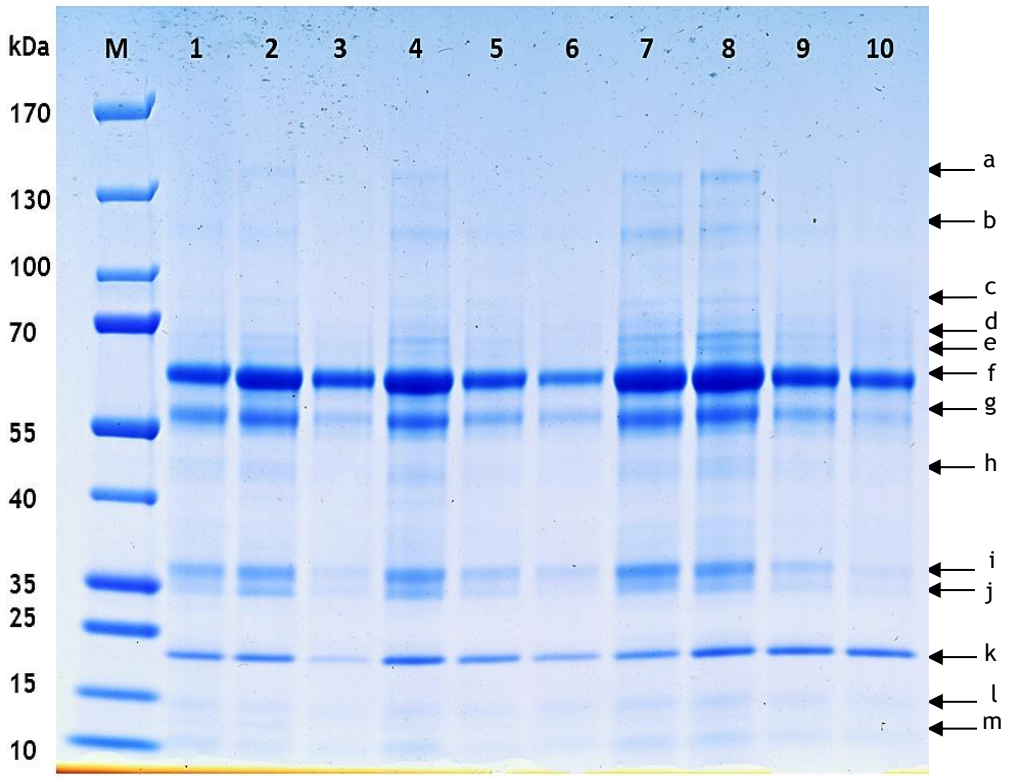


Figure 5.1: One-DE of NS from healthy animals (n=10).
Thirteen distinct protein bands labelled (a-m) were identified in Table 5.1 by MS based on the Mascot database. M= Mw marker (Precision Plus Protein Standards All Blue, Bio-Rad Inc.). Ten µl of NS sample at 1/20 dilution were loaded in well 1-10 with approximate concentration of protein loaded correspond to Table 2.4.

Band ID	Identification (UniProt Reference)	Estimated Mw (kD)	MOWSE score	Peptide matches	Coverage %
a	Alpha-2-macroglobulin	168953	3363	149	41
b	Albumin	71244	1632	66	52
c	Albumin	71244	1638	65	53
d	Lactoferrin	80113	2189	86	47
e	Serotransferrin	79856	2398	97	55
f	Albumin	71244	2880	237	71
g	Immunoglobulin heavy chain	51391	995	37	53
h	Fibrinogen beta chain	50407	831	35	44
i	Apolipoprotein A1	30258	837	50	43
j	Immunoglobulin light chain	24863	1368	51	34
k	Odorant binding protein	18447	727	85	67
l	Cytoskelatal keratin	54986	607	22	17
m	Cathelicidin	17931	490	25	44

Table 5.1: Protein with highest hits from 1-DE seperation identified by MS.
Mascot peptide database was used to identify the proteins. Data tabulated were from Mascot database result.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
a	Alpha-2-macroglobulin*

gi|408689565	alpha-2-macroglobulin variant 1 [Bos taurus]
gi|408689609	alpha-2-macroglobulin variant 23 [Bos taurus]
gi|408689603	alpha-2-macroglobulin variant 20 [Bos taurus]
gi|408689571	alpha-2-macroglobulin variant 4 [Bos taurus]
gi|1351907	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags: Precursor
gi|229552	albumin
gi|528950900	PREDICTED: alpha-2-macroglobulin [Bos taurus]
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|2194088	Chain A, Bovine Beta-Lactoglobulin, Lattice X
gi|229460	lactoglobulin beta
gi|195539527	keratin 15 [Bos taurus]
gi|2501351	RecName: Full=Serotransferrin; Short=Transferrin; AltName: Full=Beta-1 metal-binding globulin; AltName: Full=Siderophilin; Flags: Precursor
gi|27806963	alpha-S2-casein precursor [Bos taurus]
gi|530873	beta-lactoglobulin, partial [Bos taurus]
gi|148356276	keratin, type II cytoskeletal 4 [Bos taurus]
gi|432627	anti-testosterone antibody [Bos taurus]
gi|343197030	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taurus]
gi|114051856	keratin, type II cytoskeletal 7 [Bos taurus]
gi|119892108	PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus]
gi|262118301	keratin, type I cytoskeletal 14 [Bos taurus]
gi|162287381	keratin, type II cytoskeletal 73 [Bos taurus]
gi|1561571	glial fibrillary acidic protein [Bos taurus]
gi|122692325	keratin, type I cytoskeletal 28 [Bos taurus]
gi|358421411	PREDICTED: keratin, type II cytoskeletal 1b [Bos taurus]
gi|129022	RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein
gi|56710317	keratin, type II cytoskeletal 5 [Bos taurus]

Table 5.2: List of protein hits identified that have MOWSE score of >100 and peptide matches of >3 in relative to its respective protein band. Protein bands analysed were from 1-DE gel separation in Figure 5.1 and were arranged in descending order of MOWSE score. * Protein identified with the highest MOWSE score.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
b	Albumin* gi|229552 albumin gi|7547266 IgG1 heavy chain constant region [Bos taurus] gi|108750 Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine gi|296491101 TPA: ceruloplasmin (ferroxidase) [Bos taurus] gi|157954061 alpha-2-macroglobulin precursor [Bos taurus] gi|59857769 inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [Bos taurus] gi|75832116 inter-alpha-trypsin inhibitor heavy chain H4 precursor [Bos taurus] gi|91982959 immunoglobulin gamma 1 heavy chain constant region [Bos taurus] gi|440924127 alpha-2-macroglobulin [Bos taurus] gi|408689609 alpha-2-macroglobulin variant 23 [Bos taurus] gi|408689603 alpha-2-macroglobulin variant 20 [Bos taurus] gi|297474460 PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus] gi|89611 Ig gamma-2 chain C region (clone 32.2) - bovine (fragment) gi|513137425 Chain H, Crystal Structure Of Bovine Antibody Blv5b8 With Ultralong Cdr H3 gi|148238273 inter-alpha-trypsin inhibitor heavy chain H2 precursor [Bos taurus] gi|27805977 keratin, type I cytoskeletal 10 [Bos taurus] gi|509264677 immunoglobulin gamma heavy chain variable region, partial [Bos taurus] gi|1699167 IgG2a heavy chain constant region [Bos taurus] gi|119892108 PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus] gi|358421411 PREDICTED: keratin, type II cytoskeletal 1b [Bos taurus] gi|358421417 PREDICTED: keratin, type II cytoskeletal 3 [Bos taurus] gi|134085706 keratin, type II cytoskeletal 6A [Bos taurus] gi|148356276 keratin, type II cytoskeletal 4 [Bos taurus] gi|481 cytokeratin 8 (370 AA) [Bos taurus] gi|116004057 keratin, type II cytoskeletal 75 [Bos taurus] gi|56710317 keratin, type II cytoskeletal 5 [Bos taurus]

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
c	Albumin*

gi|27806815	plasminogen precursor [Bos taurus]
gi|61680007	Chain A, Crystal Structure Of Bovine Plasma Copper-Containing Amine Oxidase
gi|229552	albumin
gi|343197030	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taurus]
gi|74353860	IGL@ protein [Bos taurus]
gi|92096965	Immunoglobulin light chain, lambda gene cluster [Bos taurus]
gi|432627	anti-testosterone antibody [Bos taurus]
gi|139948632	immunoglobulin lambda-like polypeptide 1 precursor [Bos taurus]
gi|129022	RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein
gi|60592792	heat shock protein HSP 90-alpha [Bos taurus]
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|164452943	gelsolin isoform a precursor [Bos taurus]
gi|118601868	heat shock protein HSP 90-beta [Bos taurus]
gi|2501351	RecName: Full=Serotransferrin; Short=Transferrin; AltName: Full=Beta-1 metal-binding globulin; AltName: Full=Siderophilin; Flags: Precursor
gi|89611	Ig gamma-2 chain C region (clone 32.2) - bovine (fragment)
gi|504	Bovine Lactoferrin [Bos taurus]
gi|1699167	IgG2a heavy chain constant region [Bos taurus]
gi|343197026	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3c [Bos taurus]
gi|59857769	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [Bos taurus]
gi|75832116	inter-alpha-trypsin inhibitor heavy chain H4 precursor [Bos taurus]
gi|157954061	alpha-2-macroglobulin precursor [Bos taurus]
gi|408689565	alpha-2-macroglobulin variant 1 [Bos taurus]
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|296491101	TPA: ceruloplasmin (ferroxidase) [Bos taurus]
gi|83764016	prepro complement component C3 [Bos taurus]
gi|6980816	Chain C, The Crystal Structure Of Modified Bovine Fibrinogen (At ~4 Angstrom Resolution)
gi|358421409	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|343196996	immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2a [Bos taurus]
gi|114051808	complement component C7 precursor [Bos taurus]
gi|513137425	Chain H, Crystal Structure Of Bovine Antibody Blv5b8 With Ultralong Cdr H3

Table 5.2: Continued

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
d	Lactoferrin*

gi|1351907	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags: Precursor
gi|114326282	serotransferrin precursor [Bos taurus]
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|229552	albumin
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|14578599	lactoferrin [Bos taurus]
gi|34538498	immunoglobulin heavy chain constant region [Bos taurus]
gi|2232299	IgM heavy chain constant region [Bos taurus]
gi|440924127	alpha-2-macroglobulin [Bos taurus]
gi|59857769	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [Bos taurus]
gi|75832116	inter-alpha-trypsin inhibitor heavy chain H4 precursor [Bos taurus]
gi|119892108	PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus]
gi|195539527	keratin 15 [Bos taurus]
gi|27806851	lactoperoxidase precursor [Bos taurus]
gi|262118301	keratin, type I cytoskeletal 14 [Bos taurus]
gi|129022	RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein
gi|358421411	PREDICTED: keratin, type II cytoskeletal 1b [Bos taurus]
gi|122692325	keratin, type I cytoskeletal 28 [Bos taurus]
gi|148356276	keratin, type II cytoskeletal 4 [Bos taurus]
gi|6980816	Chain C, The Crystal Structure Of Modified Bovine Fibrinogen (At ~4 Angstrom Resolution)
gi|358421417	PREDICTED: keratin, type II cytoskeletal 3 [Bos taurus]
gi|114051856	keratin, type II cytoskeletal 7 [Bos taurus]
gi|432627	anti-testosterone antibody [Bos taurus]
gi|297474456	PREDICTED: keratin, type II cytoskeletal 3 [Bos taurus]
gi|116004057	keratin, type II cytoskeletal 75 [Bos taurus]
gi|76617862	PREDICTED: keratin, type II cytoskeletal 6A isoform 3 [Bos taurus]
gi|134085706	keratin, type II cytoskeletal 6A [Bos taurus]
gi|1561571	glial fibrillary acidic protein [Bos taurus]
gi|481	cytokeratin 8 (370 AA) [Bos taurus]
gi|300287	lactoferrin {internal fragment} [cattle, milk, Peptide Partial, 18 aa]
gi|157427776	keratin, type I cytoskeletal 17 [Bos taurus]
gi|115496454	keratin, type II cytoskeletal 79 [Bos taurus]
gi|3914346	RecName: Full=Polymeric immunoglobulin receptor; Short=PIgR; Short=Poly-Ig receptor; Contains: RecName: Full=Secretory component; Flags: Precursor

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
e	Serotransferrin*

gi|1351907	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags: Precursor
gi|229552	albumin
gi|408928	lactoferrin [Bos taurus]
gi|34538498	immunoglobulin heavy chain constant region [Bos taurus]
gi|3834667	immunoglobulin heavy chain constant region [Bos taurus]
gi|358420619	PREDICTED: complement C4-A isoform X2 [Bos taurus]
gi|428	unnamed protein product [Bos taurus]
gi|28592070	IgM heavy chain constant region, secretory form [Bos taurus]
gi|262050656	complement C4 precursor [Bos taurus]
gi|94981788	immunoglobulin heavy chain [Bos taurus]
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|296479365	TPA: polymeric immunoglobulin receptor precursor [Bos taurus]
gi|129022	RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein
gi|3914346	RecName: Full=Polymeric immunoglobulin receptor; Short=PIgR; Short=Poly-Ig receptor; Contains: RecName: Full=Secretory component; Flags: Precursor
gi|157954061	alpha-2-macroglobulin precursor [Bos taurus]
gi|262205546	complement C5a anaphylatoxin precursor [Bos taurus]
gi|343197018	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3b [Bos taurus]
gi|408689607	alpha-2-macroglobulin variant 22 [Bos taurus]
gi|41386699	heat shock-related 70 kDa protein 2 [Bos taurus]
gi|1699167	IgG2a heavy chain constant region [Bos taurus]
gi|157280001	heparin cofactor 2 precursor [Bos taurus]
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|59857769	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) [Bos taurus]

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
f	Albumin*

gi|61680007	Chain A, Crystal Structure Of Bovine Plasma Copper-Containing Amine Oxidase
gi|229552	albumin
gi|27806815	plasminogen precursor [Bos taurus]
gi|528995966	PREDICTED: primary amine oxidase, lung isozyme isoform X1 [Bos taurus]
gi|77736201	gelsolin isoform b [Bos taurus]
gi|60592792	heat shock protein HSP 90-alpha [Bos taurus]
gi|129022	RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein
gi|118601868	heat shock protein HSP 90-beta [Bos taurus]
gi|7547266	IgG1 heavy chain constant region [Bos taurus]
gi|89611	Ig gamma-2 chain C region (clone 32.2) - bovine (fragment)
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|343197030	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taurus]
gi|151556360	Unknown (protein for MGC:159378) [Bos taurus]
gi|74353860	IGL@ protein [Bos taurus]
gi|92096965	Immunoglobulin light chain, lambda gene cluster [Bos taurus]
gi|2501351	RecName: Full=Serotransferrin; Short=Transferrin; AltName: Full=Beta-1 metal-binding globulin; AltName: Full=Siderophilin; Flags: Precursor
gi|91982959	immunoglobulin gamma 1 heavy chain constant region [Bos taurus]
gi|1699167	IgG2a heavy chain constant region [Bos taurus]
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|513137425	Chain H, Crystal Structure Of Bovine Antibody Blv5b8 With Ultralong Cdr H3
gi|296476451	TPA: primary amine oxidase, liver isozyme-like [Bos taurus]
gi|343197018	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3b [Bos taurus]
gi|296491101	TPA: ceruloplasmin (ferroxidase) [Bos taurus]
gi|343196996	immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2a [Bos taurus]
gi|83764016	prepro complement component C3 [Bos taurus]
gi|75832056	apolipoprotein A-I preproprotein [Bos taurus]
gi|6980816	Chain C, The Crystal Structure Of Modified Bovine Fibrinogen (At ~4 Angstrom Resolution)
gi|528948723	PREDICTED: keratin, type II cytoskeletal 2 epidermal isoform X2 [Bos taurus]
gi|296475269	TPA: ZBTB42 protein-like [Bos taurus]
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|114051808	complement component C7 precursor [Bos taurus]
gi|27806941	alpha-1-antiproteinase precursor [Bos taurus]

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
g	Immunoglobulin heavy chain*

gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|229552	albumin
gi|89611	Ig gamma-2 chain C region (clone 32.2) - bovine (fragment)
gi|1699167	IgG2a heavy chain constant region [Bos taurus]
gi|397740864	vitamin D binding protein [Bos taurus]
gi|77736341	antithrombin-III precursor [Bos taurus]
gi|513137425	Chain H, Crystal Structure Of Bovine Antibody Blv5b8 With Ultralong Cdr H3
gi|509264677	immunoglobulin gamma heavy chain variable region, partial [Bos taurus]
gi|164450479	kininogen-2 isoform I precursor [Bos taurus]
gi|38683423	endopin 2B [Bos taurus]
gi|1293592	immunoglobulin heavy chain variable region, partial [Bos taurus]
gi|27806751	alpha-2-HS-glycoprotein precursor [Bos taurus]
gi|513137421	Chain H, Crystal Structure Of Bovine Antibody Blv1h12 With Ultralong Cdr H3
gi|114051361	selenium-binding protein 1 [Bos taurus]
gi|27806321	retinal dehydrogenase 1 [Bos taurus]
gi|75832056	apolipoprotein A-I preproprotein [Bos taurus]
gi|77735387	fetuin-B precursor [Bos taurus]
gi|1293600	immunoglobulin heavy chain variable region, partial [Bos taurus]
gi|27806941	alpha-1-antiproteinase precursor [Bos taurus]
gi|509264987	immunoglobulin alpha heavy chain variable region, partial [Bos taurus]
gi|4063715	immunoglobulin IgA heavy chain constant region [Bos taurus]

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
h	Fibrinogen beta chain* gi|148744172 Actin, beta [Bos taurus] gi|156120479 fructose-bisphosphate aldolase A [Bos taurus] gi|22655316 beta-actin [Bos taurus] gi|95769010 serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1 [Bos taurus] gi|1699167 IgG2a heavy chain constant region [Bos taurus] gi|4501881 actin, alpha skeletal muscle [Homo sapiens] gi|528976936 PREDICTED: apolipoprotein A-IV isoform X1 [Bos taurus] gi|89611 Ig gamma-2 chain C region (clone 32.2) - bovine (fragment) gi|87196501 alpha-enolase [Bos taurus] gi|194676388 PREDICTED: actin, beta-like 2 isoform X1 [Bos taurus] gi|94966811 alpha-1-acid glycoprotein precursor [Bos taurus] gi|7547266 IgG1 heavy chain constant region [Bos taurus] gi|129022 RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein gi|513137425 Chain H, Crystal Structure Of Bovine Antibody Biv5b8 With Ultralong Cdr H3 gi|2501351 RecName: Full=Serotransferrin; Short=Transferrin; AltName: Full=Beta-1 metal-binding globulin; AltName: Full=Siderophilin; Flags: Precursor gi|151557009 SERPINB4 protein [Bos taurus] gi|119924103 PREDICTED: serpin B3-like [Bos taurus] gi|151556360 Unknown (protein for MGC:159378) [Bos taurus] gi|296477761 TPA: Fc fragment of IgG binding protein-like [Bos taurus] gi|27805977 keratin, type I cytoskeletal 10 [Bos taurus] gi|343197030 immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taurus] gi|77736349 beta-enolase [Bos taurus] gi|114053183 serum paraoxonase/arylesterase 1 precursor [Bos taurus] gi|332078523 serpin peptidase inhibitor, clade B like [Bos taurus] gi|995923 immunoglobulin heavy chain variable region, partial [Bos taurus] gi|6980816 Chain C, The Crystal Structure Of Modified Bovine Fibrinogen (At ~4 Angstrom Resolution)

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
i	Apolipoprotein A1*

gi|92096965	Immunoglobulin light chain, lambda gene cluster [Bos taurus]
gi|74353860	IGL@ protein [Bos taurus]
gi|151556360	Unknown (protein for MGC:159378) [Bos taurus]
gi|432627	anti-testosterone antibody [Bos taurus]
gi|343197030	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taur
gi|15088675	immunoglobulin lambda light chain [Bos taurus]
gi|1351907	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags
gi|343197004	immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2b [Bos taur
gi|229552	albumin
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|115545495	IGK protein [Bos taurus]
gi|29135329	glutathione S-transferase P [Bos taurus]
gi|7547266	IgG1 heavy chain constant region [Bos taurus]
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|28461273	glutathione S-transferase Mu 1 [Bos taurus]
gi|2323374	immunoglobulin light chain variable region [Bos taurus]
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|296478325	TPA: immunoglobulin lambda-6a light chain variable region-like [Bos taurus]
gi|119892108	PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus]
gi|358421411	PREDICTED: keratin, type II cytoskeletal 1b [Bos taurus]
gi|114053087	glutathione S-transferase mu 3 [Bos taurus]
gi|975846	immunoglobulin lambda light chain variable region, partial [Bos taurus]
gi|2323400	immunoglobulin light chain variable region [Bos taurus]
gi|262118301	keratin, type I cytoskeletal 14 [Bos taurus]
gi|358414626	PREDICTED: neutrophil gelatinase-associated lipocalin isoformX2 [Bos taurus]
gi|297465561	PREDICTED: glutathione S-transferase Mu 1 isoform X1 [Bos taurus]
gi|148356276	keratin, type II cytoskeletal 4 [Bos taurus]
gi|89611	Ig gamma-2 chain C region (clone 32.2) - bovine (fragment)

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
j	Immunoglobulin light chain*

gi|513137422	Chain L, Crystal Structure Of Bovine Antibody Blv1h12 With Ultralong Cdr H3
gi|151556360	Unknown (protein for MGC:159378) [Bos taurus]
gi|432627	anti-testosterone antibody [Bos taurus]
gi|148878143	Unknown (protein for MGC:159411) [Bos taurus]
gi|15088675	immunoglobulin lambda light chain [Bos taurus]
gi|75832056	apolipoprotein A-I preproprotein [Bos taurus]
gi|343197030	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taurus]
gi|343196996	immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2a [Bos taurus]
gi|343197004	immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2b [Bos taurus]
gi|115545495	IGK protein [Bos taurus]
gi|343197008	immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2c [Bos taurus]
gi|2323374	immunoglobulin light chain variable region [Bos taurus]
gi|1351907	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags: Precursor
gi|2323404	immunoglobulin light chain variable region [Bos taurus]
gi|343197018	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3b [Bos taurus]
gi|2323400	immunoglobulin light chain variable region [Bos taurus]
gi|229552	albumin
gi|2323394	immunoglobulin light chain variable region [Bos taurus]
gi|27807167	peroxiredoxin-6 [Bos taurus]
gi|508836	anti-respiratory syncytial virus Ig lambda chain V region, partial [Bos taurus]
gi|2323378	immunoglobulin light chain variable region [Bos taurus]
gi|108750	Ig heavy chain precursor (B/MT.4A.17.H5.A5) - bovine
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|310893441	immunoglobulin light chain [Bos taurus]
gi|296478325	TPA: immunoglobulin lambda-6a light chain variable region-like [Bos taurus]
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|119892108	PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus]
gi|61888856	triosephosphate isomerase [Bos taurus]
gi|114053087	glutathione S-transferase mu 3 [Bos taurus]

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
k	Odorant binding protein* gi|17943132 Chain A, Crystal Structure Of Bovine Obp Complexed With Aminoanthracene gi|170784958 Chain A, Bovine Odorant Binding Protein Deswapped Triple Mutant gi|297474460 PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus] gi|27805977 keratin, type I cytoskeletal 10 [Bos taurus] gi|432627 anti-testosterone antibody [Bos taurus] gi|119892108 PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus] gi|358421411 PREDICTED: keratin, type II cytoskeletal 1b [Bos taurus] gi|343197030 immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d [Bos taurus] gi|76677514 PREDICTED: C4b-binding protein alpha chain [Bos taurus] gi|4502201 ADP-ribosylation factor 1 [Homo sapiens] gi|358421417 PREDICTED: keratin, type II cytoskeletal 3 [Bos taurus] gi|76617862 PREDICTED: keratin, type II cytoskeletal 6A isoform 3 [Bos taurus] gi|56710317 keratin, type II cytoskeletal 5 [Bos taurus] gi|481 cytokeratin 8 (370 AA) [Bos taurus] gi|116004057 keratin, type II cytoskeletal 75 [Bos taurus] gi|296487895 TPA: mCG144546-like [Bos taurus] gi|148356276 keratin, type II cytoskeletal 4 [Bos taurus] gi|77736007 ADP-ribosylation factor 4 [Bos taurus]

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)			
l	keratin*	gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]	Cytoskeletal
		gi|119892108	PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus]	
		gi|195539527	keratin 15 [Bos taurus]	
		gi|262118301	keratin, type I cytoskeletal 14 [Bos taurus]	
		gi|528948721	PREDICTED: keratin, type II cytoskeletal 1b [Bos taurus]	
		gi|148356276	keratin, type II cytoskeletal 4 [Bos taurus]	
		gi|27806751	alpha-2-HS-glycoprotein precursor [Bos taurus]	
		gi|165973998	protein S100-A8 [Bos taurus]	
		gi|528996272	PREDICTED: keratin, type I cytoskeletal 42-like isoform X1 [Bos taurus]	
		gi|157427776	keratin, type I cytoskeletal 17 [Bos taurus]	
		gi|358421417	PREDICTED: keratin, type II cytoskeletal 3 [Bos taurus]	
		gi|1351907	RecName: Full=Serum albumin; AltName: Full=BSA; AltName: Allergen=Bos d 6; Flags: Precursor	
		gi|76617862	PREDICTED: keratin, type II cytoskeletal 6A isoform 3 [Bos taurus]	
		gi|114051856	keratin, type II cytoskeletal 7 [Bos taurus]	
		gi|34365785	resistin precursor [Bos taurus]	
		gi|162287381	keratin, type II cytoskeletal 73 [Bos taurus]	
		gi|300797165	keratin, type II cuticular Hb4 [Bos taurus]	
		gi|118151000	keratin, type II cytoskeletal 80 [Bos taurus]	
		gi|83764016	prepro complement component C3 [Bos taurus]	
		gi|1438768	cornea-associated antigen, CO-Ag=calgranulin C homolog [cattle, corneal stroma, Peptide, 70 aa]	
		gi|229552	albumin	
		gi|296487895	TPA: mCG144546-like [Bos taurus]	
		gi|56710317	keratin, type II cytoskeletal 5 [Bos taurus]	

Table 5.2: Continued.

Band ID	List of protein hits identified having MOWSE score of >100 and peptide matches of >3 (UniProt Reference)
m	Cathelicidin*

gi|528925568	PREDICTED: zymogen granule protein 16 homolog B [Bos taurus]
gi|296473587	TPA: pancreatic adenocarcinoma upregulated factor-like [Bos taurus]
gi|296473588	TPA: HRPE773-like [Bos taurus]
gi|129022	RecName: Full=Odorant-binding protein; Short=OBP; AltName: Full=Olfactory mucosa pyrazine-binding protein
gi|27819608	hemoglobin subunit beta [Bos taurus]
gi|122539	RecName: Full=Hemoglobin subunit beta-A; AltName: Full=Beta-A-globin; AltName: Full=Hemoglobin beta-A chain
gi|118150406	secretoglobin family 1D member precursor [Bos taurus]
gi|155372309	mammaglobin-A precursor [Bos taurus]
gi|162797	beta-casein precursor [Bos taurus]
gi|27806789	transthyretin precursor [Bos taurus]
gi|576142	Chain A, A Novel Allosteric Mechanism In Haemoglobin. Structure Of Bovine Deoxyhaemoglobin, Absence Of Specific Chloride- Binding Sites And Origin Of The Chloride-Linked Bohr Effect
gi|297474460	PREDICTED: keratin, type II cytoskeletal 1 [Bos taurus]
gi|27807343	cathelicidin-2 precursor [Bos taurus]
gi|148356276	keratin, type II cytoskeletal 4 [Bos taurus]
gi|27807337	cathelicidin-4 precursor [Bos taurus]
gi|62751593	profilin-1 [Bos taurus]
gi|27805977	keratin, type I cytoskeletal 10 [Bos taurus]
gi|114051856	keratin, type II cytoskeletal 7 [Bos taurus]
gi|162287381	keratin, type II cytoskeletal 73 [Bos taurus]
gi|300797165	keratin, type II cuticular Hb4 [Bos taurus]
gi|164450479	kininogen-2 isoform I precursor [Bos taurus]
gi|432627	anti-testosterone antibody [Bos taurus]
gi|119892108	PREDICTED: keratin, type II cytoskeletal 2 epidermal [Bos taurus]

Table 5.2: Continued.

5.3.2 Two-DE separation from six individual samples and analysis of protein spots expressions

All 6 individual gel images from healthy animal are shown in Figure 5.2. Variation of protein spots intensities across 6 individual 2-DE gels were compared using SameSpots 2D gel image analysis software. Two-DE image analysis showed 60 differentially expressed spots with ANOVA P ≤0.05 and 60 spots with fold change ≥2, with power >0.8 (Figure 5.3). The variation between the 6 individuals for selected peaks is described in section 5.3.5 after the identity of protein was determined by MS of spots from the 2-DE gel of the pooled sample.

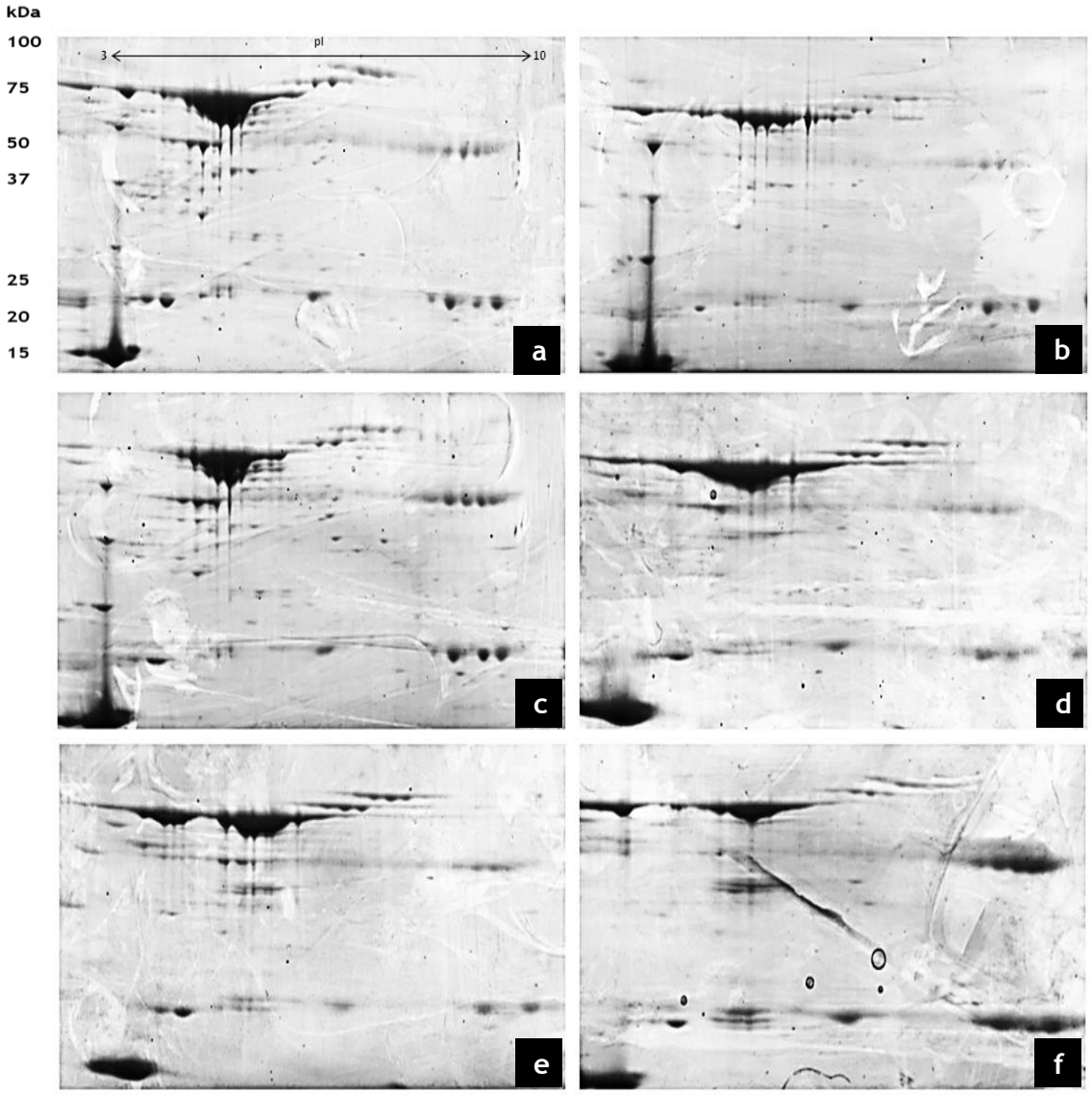


Figure 5.2: Sample images of healthy NS proteome on 2-DE gels from 6 different animals (a-f).
Protein separation was identical to pooled sample however the protein spots intensity for Ig heavy and light chains were lighter in individual samples. No obvious variation of protein spots distribution or expression were observed within the individual samples

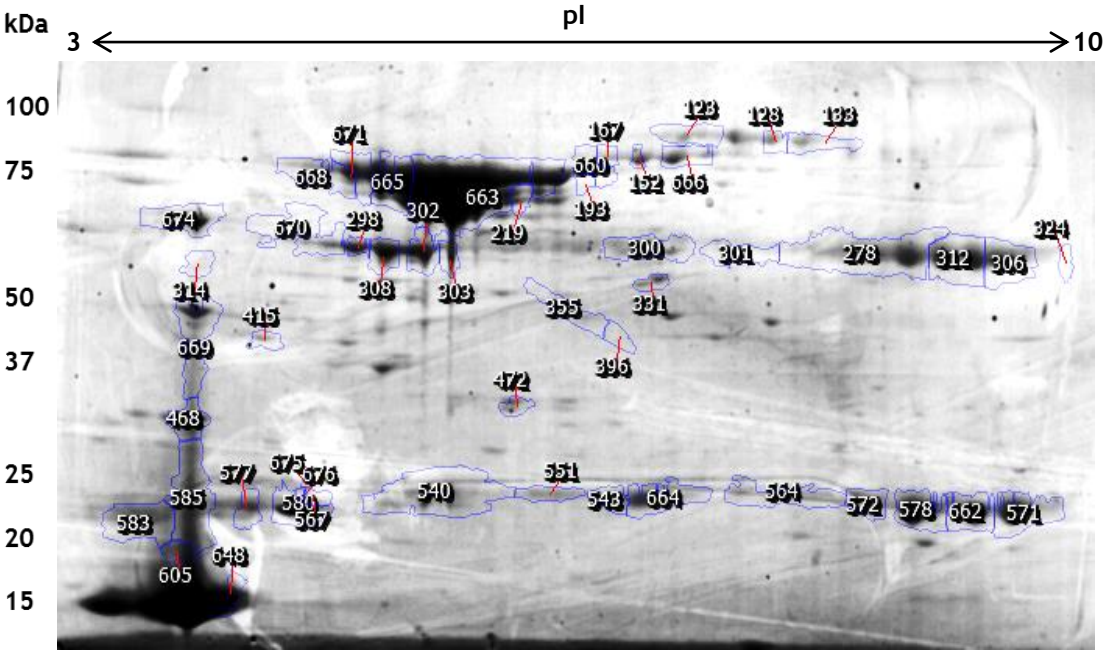


Figure 5.3: Reference image of 2-DE map of differentially expressed spots across the 6 different NS collected from healthy cattle.
Representative of the composite for 2-DE map of NS obtained by performing the first dimension (IEF) on IPG strips pH 3–10 and the second dimension on 4–12% gradient SDS-PAGE gels. The protein spots were visualized by Coomassie blue staining. Mr standard values are indicated on the left.

5.3.3 Two-DE on pooled nasal secretion samples

Two-dimensional electrophoresis separation was carried out on pooled NS samples in triplicate. Initial 2-DE image visual analysis showed NS protein were well separated across the gel having major protein spots at 75 kDa, 60 kDa and 25 kDa marked as x, y and z respectively (Figure 5.4) . Preliminary presumption of protein identification by comparison to 2DE gels of bovine serum (Skrzypczak et al., 2001) were made with these 3 major protein spots which are x = albumin; y = Ig heavy chain and z = Ig light chain. Replicates of the 2-DE gel of pooled NS samples show no significant difference in protein spots distribution.

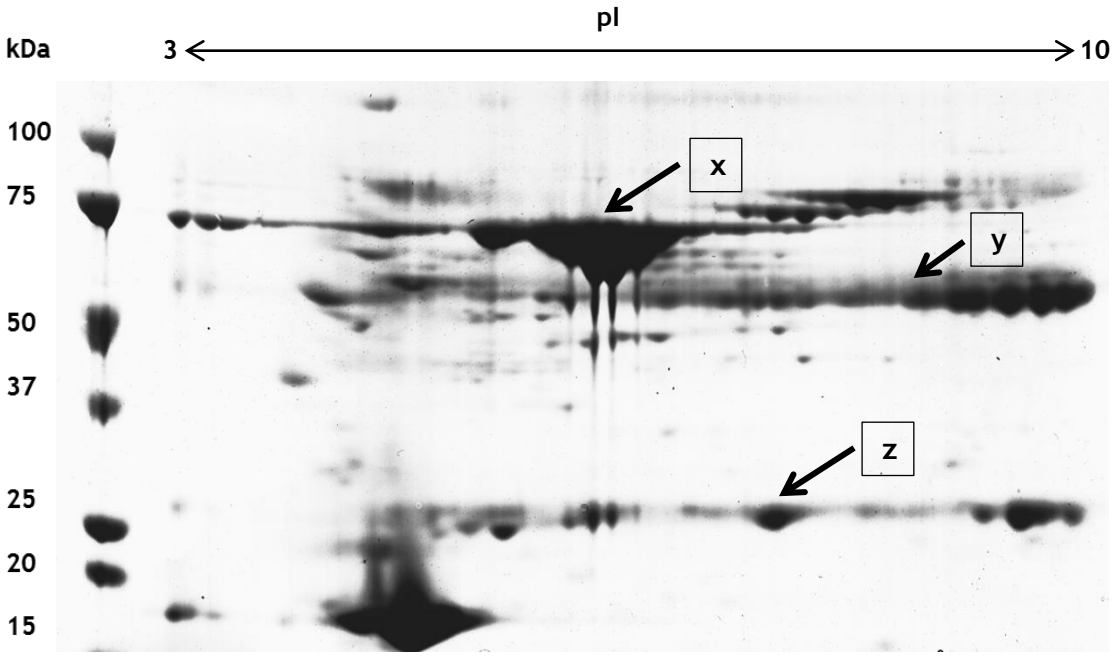


Figure 5.4: Protein spots of pooled bovine NS from healthy animal identified on 2-DE. Preliminary presumption were made at that time indicating major NS protein such as x=albumin; y=Ig heavy chain and z=Ig light chain.

5.3.4 Identification of major proteins in bovine nasal secretion after 2-DE

All major high abundance protein spots were selected for protein identification. A total of 40 protein spots were selected and analysed for protein identification by MS. All protein spots that were identified by MS were later grouped into ten major proteins (Figure 5.5). Following that, the 10 major proteins were further grouped according to the source of production mainly glandular, vascular and cellular proteins. The total protein spots identified by MS were listed in Table 5.3 and data were also presented in percentage as shown in Figure 5.6. Selection criteria in the list were Mascot score higher than 100 and sequence coverage over 10%.

Distinct functional categories to which these proteins can be ascribed are indicated in Figure 5.7. However, the results show the percentages of the total types of nasal protein present in the secretion and do not represent their respective concentration in the NS. Polypeptides involved in innate (34%) and acquired immunity (20%) system were major components. The proteins identified were also categorized according to their source of origin mainly vascular, cellular, glandular and cytoskel etal derives proteins based on protein ontology search in NCBI. Vascular proteins (74% of all proteins identified) may be derived from plasma exudation from the local blood vessels that are present in the nasal cavity, turnover of epithelial goblet and ciliated cells (cytoskeletal proteins, 11%), cellular proteins (6%) and exocytosis from glands (glandular proteins, 9%) as represented in Figure 5.8.

Glandular proteins constitute 9% out of the 40 total protein spots that were selected for having significant expression changes and identification. Proteins categorized in this group were the odorant binding protein and lactoferrin. Vascular proteins are the most abundant protein present in bovine NS, comprising 85% of all the protein detected. These proteins commonly derived through plasma extravasation from the superficial fenestrated capillaries and post capillary venous flow which exudes across the nasal epithelium provides the constituent of NS. Consequently this process contributes the albumin, immunoglobulins mainly monomeric IgA, IgG, Ig heavy chains and light chains, serotransferrin and apolipoprotein-A1 were detected frequently having the same concentrations as their plasma.

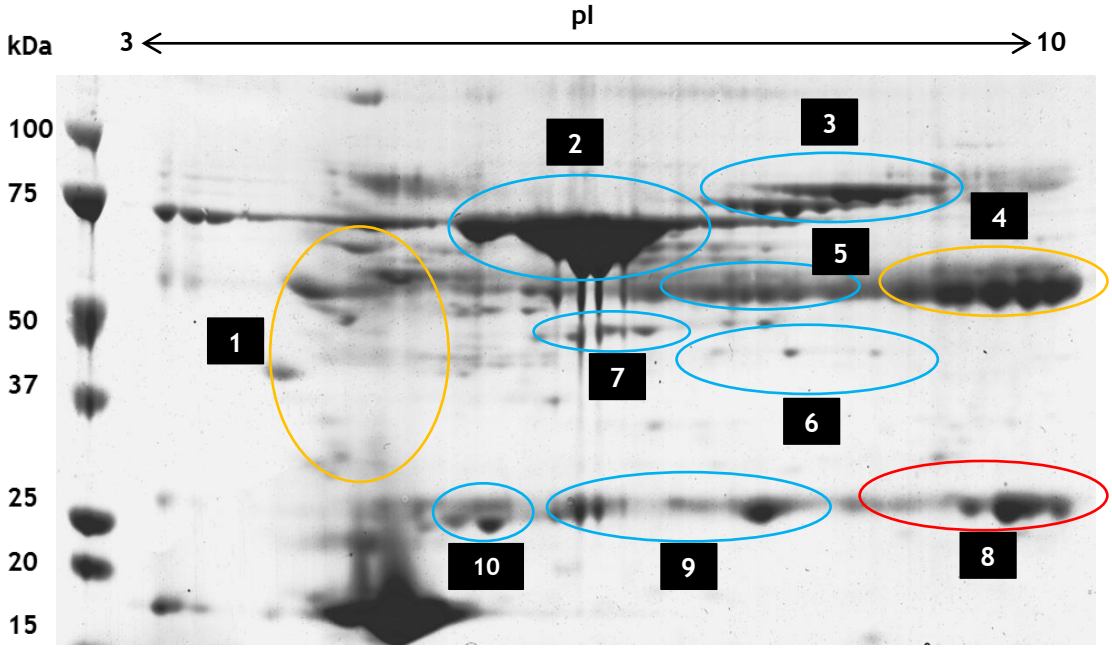


Figure 5.5: Two-DE with major spots of high abundance proteins selected and identified with MS.
Protein spots were individually identified as shown in Table 5.3 and colour grouped (circled) according to their origin. Yellow=glandular proteins; blue=vascular proteins and red=cellular proteins.

Protein spot no.*	Protein identification (UniProt reference)	Theoretical pI	Theoretical Mw (Da)	MOWSE Score	Peptide Matches	% coverage
123	Serotransferrin (TRFE_BOVIN)	6.75	135925	300	45	13
128	Serotransferrin variant 1 (Q2HJF0_BOVIN)	7.46	79870	1394	102	11
133	Serotransferrin precursor (G3X6N3_BOVIN)	8.01	119410	129	36	14
152	Serum albumin precursor (AMBP_BOVIN)	5.26	68772	101	31	10
219	Serum albumin precursor (VTDB_BOVIN)	5.60	66432	240	68	14
278	Ig heavy chain precursor (B/MT.4A.17.H5.A5) – bovine (O46780_BOVIN)	6.07	49981	166	77	19
298	Apolipoprotein A-I precursor (V6F9A2_BOVIN)	4.21	128432	107	21	18
300	Ig heavy chain (Q45UT4_BOVIN)	6.35	51309	621	38	23
301	Fibrinogen beta chain (FIBB_BOVIN)	6.66	50993	117	23	31
302	Serum albumin precursor variant 4 (ALBU_BOVIN)	5.75	70951	101	27	10
303	Complement C3 precursor (G3X7A5_BOVIN)	4.54	185047	108	36	10
306	Lactoferrin (B9VPZ5_BOVIN)	8.69	78056	103	20	14
308	Complement C3 (CO3_BOVIN)	4.97	175007	115	27	16
312	Lactoferrin (TRFL_BOVIN)	8.50	80002	129	32	18
314	Odorant binding protein precursor (Q0IIA2_BOVIN)	3.98	55030	107	26	21
331	Ig heavy chain (Q2TBV8_BOVIN)	5.74	57902	121	45	30
396	Fibrinogen beta chain (F1MAV0_BOVIN)	8.45	45650	114	29	16
415	Albumin precursor (ALBU_BOVIN)	4.23	31820	140	33	10
468	Serum albumin precursor variant 7 (VTDB_BOVIN)	3.82	30071	139	42	12
472	Complement C3 precursor (CO3_BOVIN)	4.68	63048	118	18	10
540	Apolipoprotein A1 (APOA1_BOVIN)	5.71	30276	1296	77	36
543	Immunoglobulin light chain (H2EQP9_BOVIN)	7.54	24536	118	28	14
567	Apolipoprotein A1(APOA1_BOVIN)	5.36	27549	130	60	16
571	Glutathione S-transferase (GSTA2_BOVIN)	8.33	9048	118	21	14
572	Immunoglobulin light chain, lambda gene cluster (Q1RMN8_BOVIN)	7.54	24536	189	23	16
577	Serum albumin precursor (AMBP_BOVIN)	4.53	2122	126	17	12

578	Gluthathione S-transferase (GSTP1_BOVIN)	7.30	23481	525	26	11
580	Apolipoprotein A1 (APOA1_BOVIN)	5.57	28432	179	32	18
583	Odorant binding protein (OBP_BOVIN)	3.57	2765	457	144	21
585	Keratin, type 1 cytoskelatal 10- *Bos taurus* (K1C10_BOVIN)	3.79	18503	187	33	16
605	Keratin, type II cytoskeletal 8 – *Bos taurus* (K2C8_BOVIN)	3.79	18509	233	48	12
662	Surrogate light chain (O77624_BOVIN)	8.02	21114	170	22	12
660	Serum albumin precursor (ALBU_BOVIN)	5.69	79901	1391	121	28
663	Serum albumin (ALBU_BOVIN)	5.60	79870	2568	163	30
664	immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3d, partial - *Bos taurus* (H2EQP6_BOVIN)	4.63	19711	230	22	16
666	Lactoferrin (Q6LBN7_BOVIN)	7.67	76143	301	40	14
668	Serum albumin precursor (Q5DM41_BOVIN)	5.00	76020	124	27	10
670	Serum albumin precursor (AMBP_BOVIN)	4.81	69526	179	86	13
671	Serum albumin precursor (ALBU_BOVIN)	5.53	22233	137	20	11
674	Odorant binding protein (OBP_BOVIN)	4.91	18503	600	135	19

Table 5.3: Protein spot identifications (n=40) from 2-DE conducted in section 5.3.2.
List of proteins identified by MS, together with theoretical isoelectric point (pI), molecular weights (Mw), MOWSE score, peptide matches and percentage of coverage. * Protein spots selected from 2-DE separation in Figure 5.3.

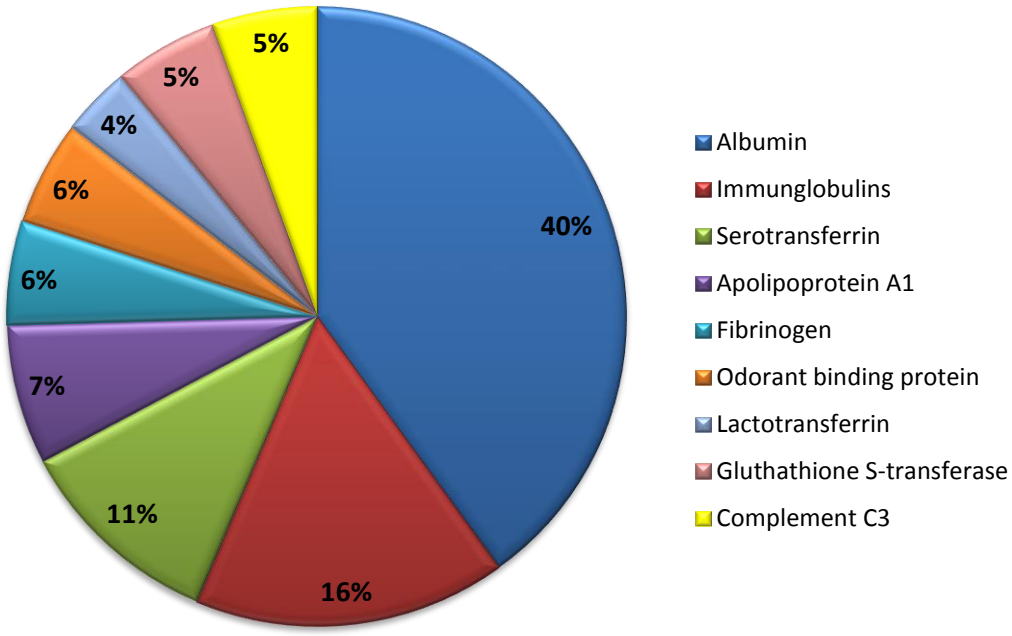


Figure 5.6: Major high abundance protein spots selected and identified using MS (n=40). Data presented in percentage.

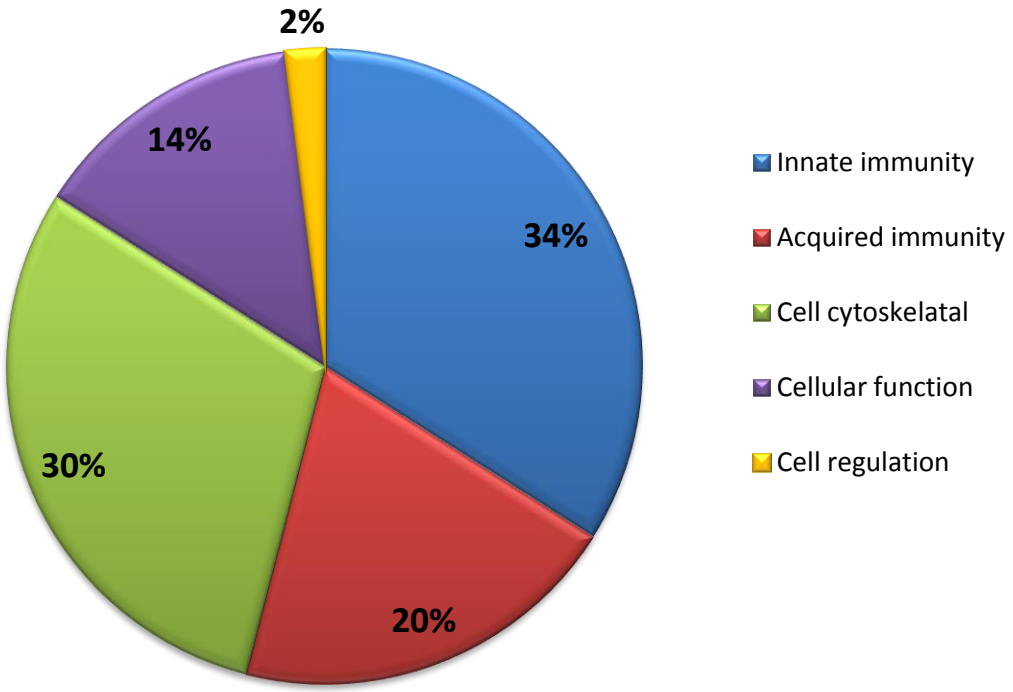


Figure 5.7: Pie chart showing the classification of the proteins identified in bovine NS (n=40) according to their biological function based on protein ontology search in NCBI.

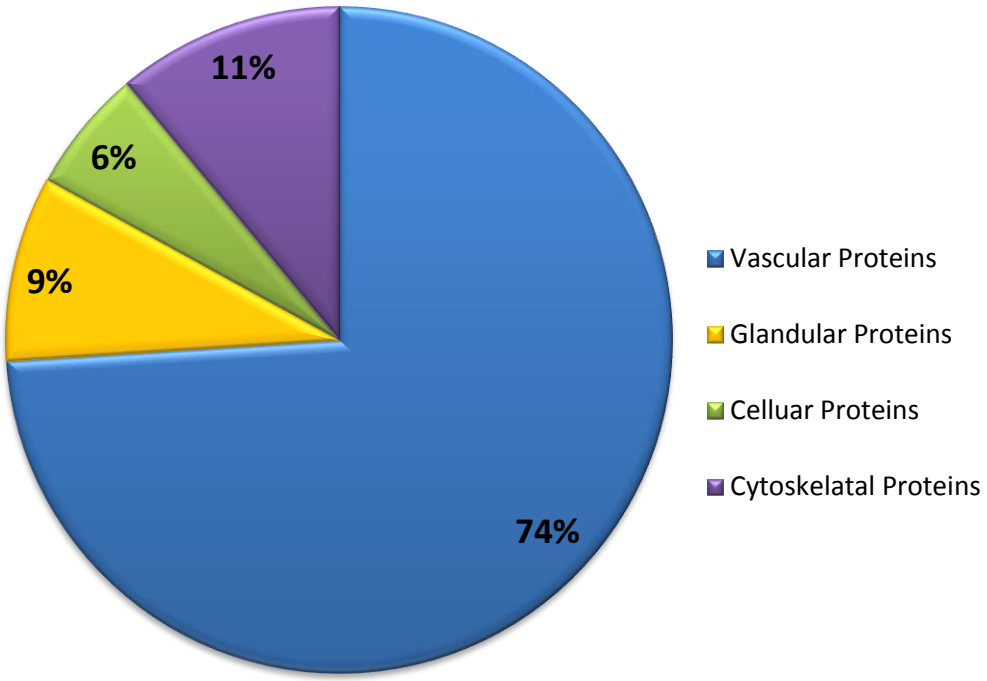


Figure 5.8: Percentage of proteins (n=40) identified by MS further categorized according to their source based on protein ontology search in NCBI.

5.3.5 Variation of protein spot intensity across six healthy nasal secretion samples

Figure 5.9 to 5.18 show the image analysis of protein spots that was carried out using SameSpots 2-DE gel image analysis software to quantify variation of protein spot intensities of NS across 6 healthy individual cattle (section 5.3.2) with mean and range of the protein spot intensity given for the 10 major proteins identified in section 5.3.4. Protein spot intensities were given in normalized log volume but were transformed into actual value of data (intensity unit) and compared in Figure 5.19. The median and range for protein spot intensity for all major protein were presented in Figure 5.20.

The mean (±SD) for the actual value of protein intensity for all the major proteins were spot 663:albumin (8.02 ± 1.4), spot 540:apolipoprotein A1 (5.84 ± 3.9), spot 308:complement C3 (4.38 ± 1.8), spot 301:fibrinogen beta chain (2.86 ± 0.4), spot 571:glutathione S-transferase (1.10 ± 0.2), spot 583:odorant binding protein (6.13 ± 2.5), spot 300:Ig heavy chain (4.37 ± 1.0), spot 543:Ig light chain (6.71 ± 2.8), spot 128:serotransferrin (2.85 ± 0.6) and spot 666:lactoferrin (1.94 ± 0.8).

Albumin

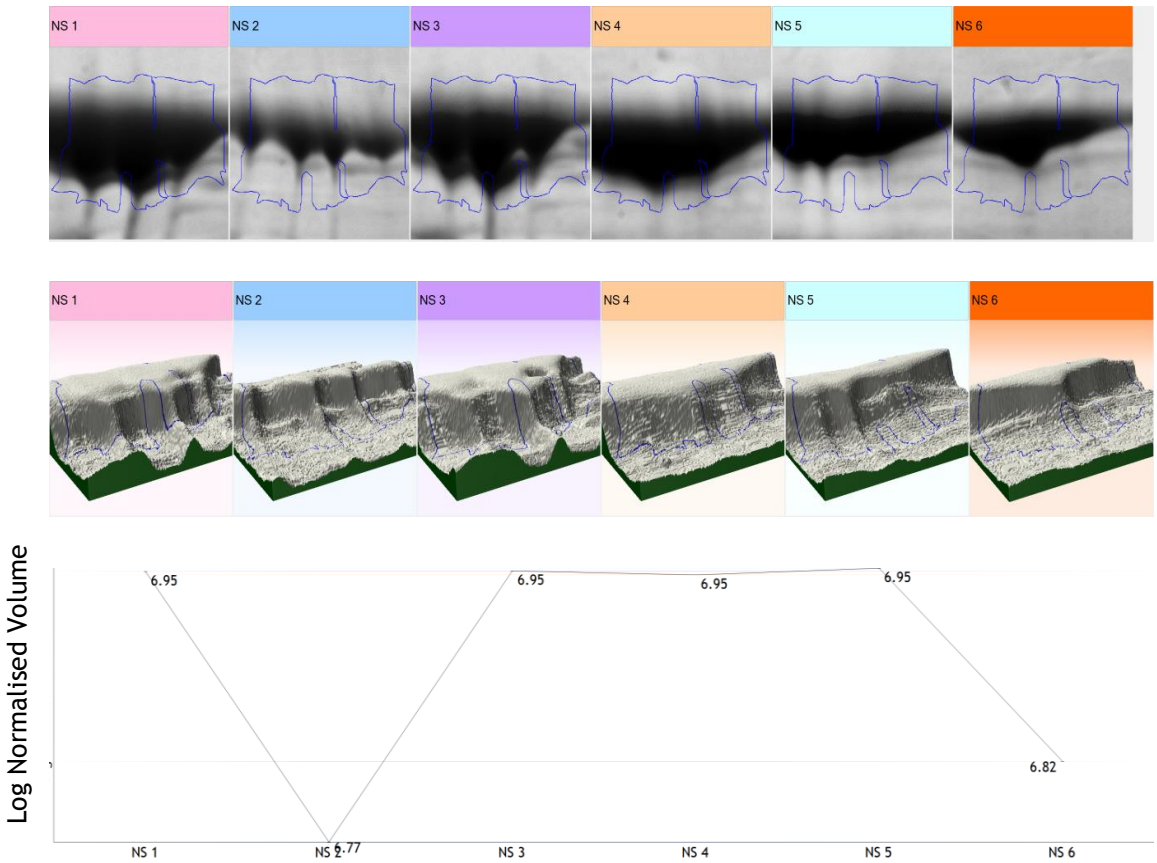


Figure 5.9: Variation in albumin spot intensity between individual NS samples. The median of albumin protein spot intensity between 6.95 different NS is 6.9 ranging from 6.77 – 6.95.

Apolipoprotein A1

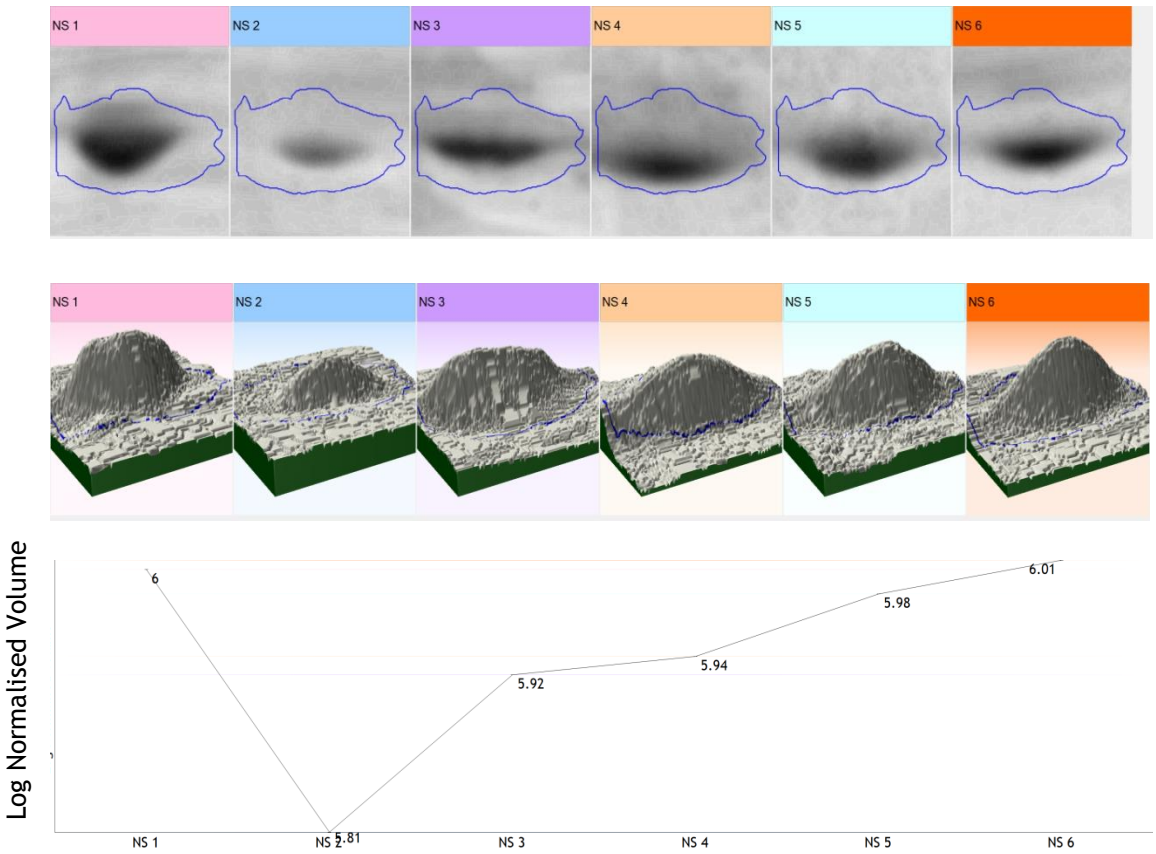


Figure 5.10: Variation in apolipoprotein A1 spot intensity between individual NS samples. The median of apolipoprotein A1 spot intensity between 6 different NS is 5.96 ranging from 5.81 – 6.01.

Complement C3

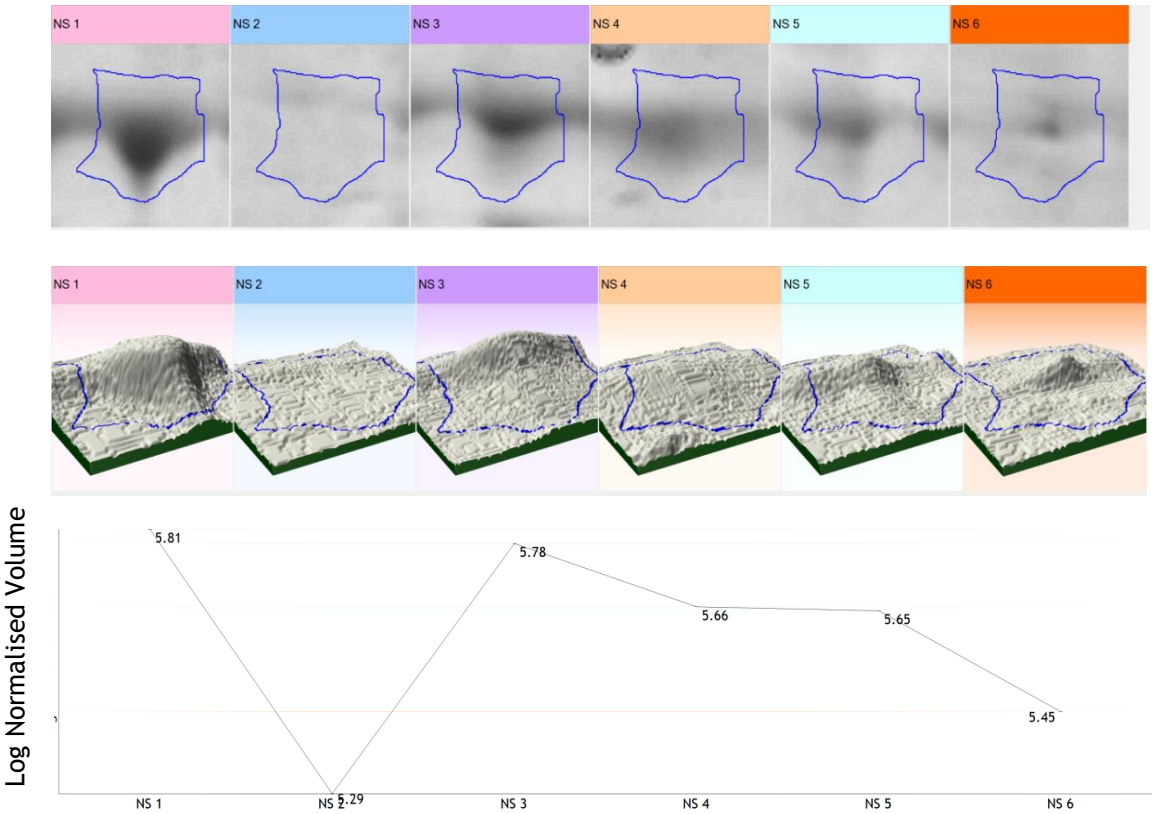


Figure 5.11: Variation in complement C3 spot intensity between individual NS samples. The median of complement C3 protein spot intensity between 6 different NS is 5.66 ranging from 5.29 – 5.81.

Fibrinogen beta chain

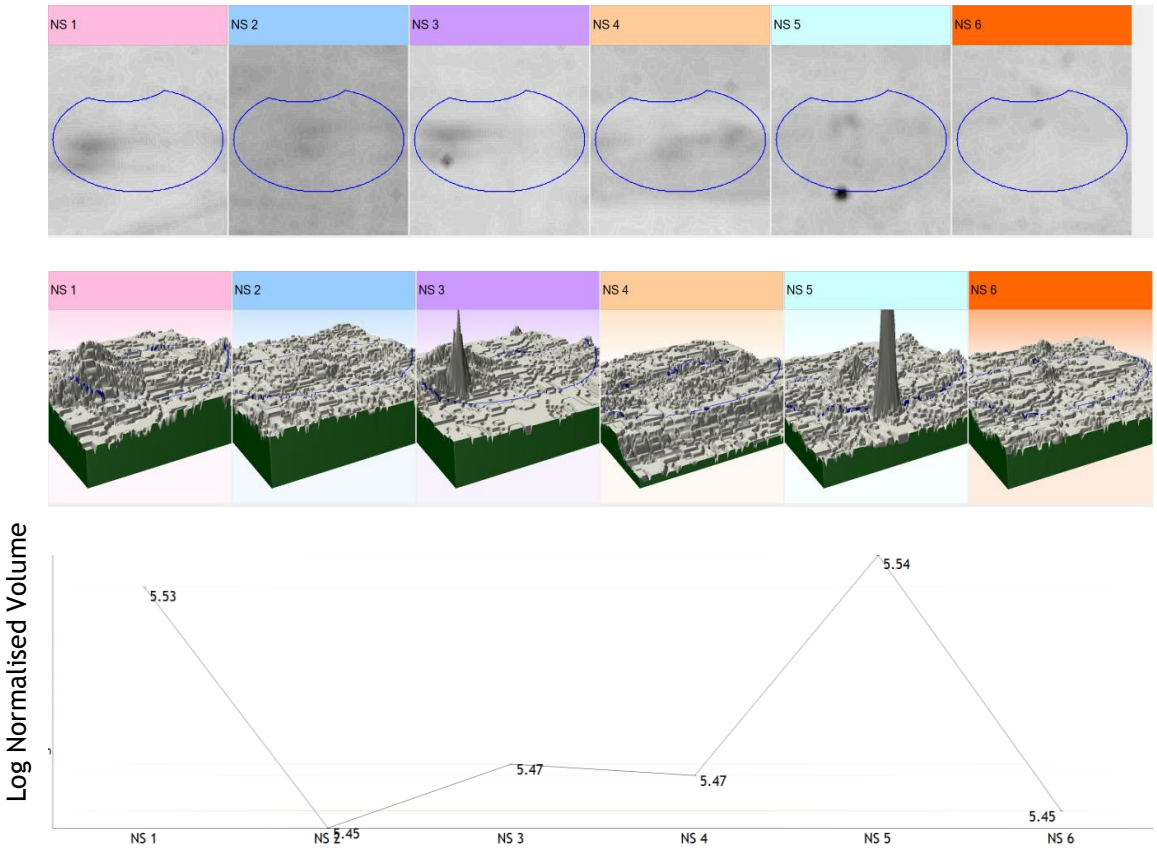


Figure 5.12: Variation in fibrinogen beta chain spot intensity between individual NS samples. The median of fibrinogen beta chain protein spot intensity between 6 different NS is 5.47 ranging from 5.45 – 5.54.

Glutathione S-transferase

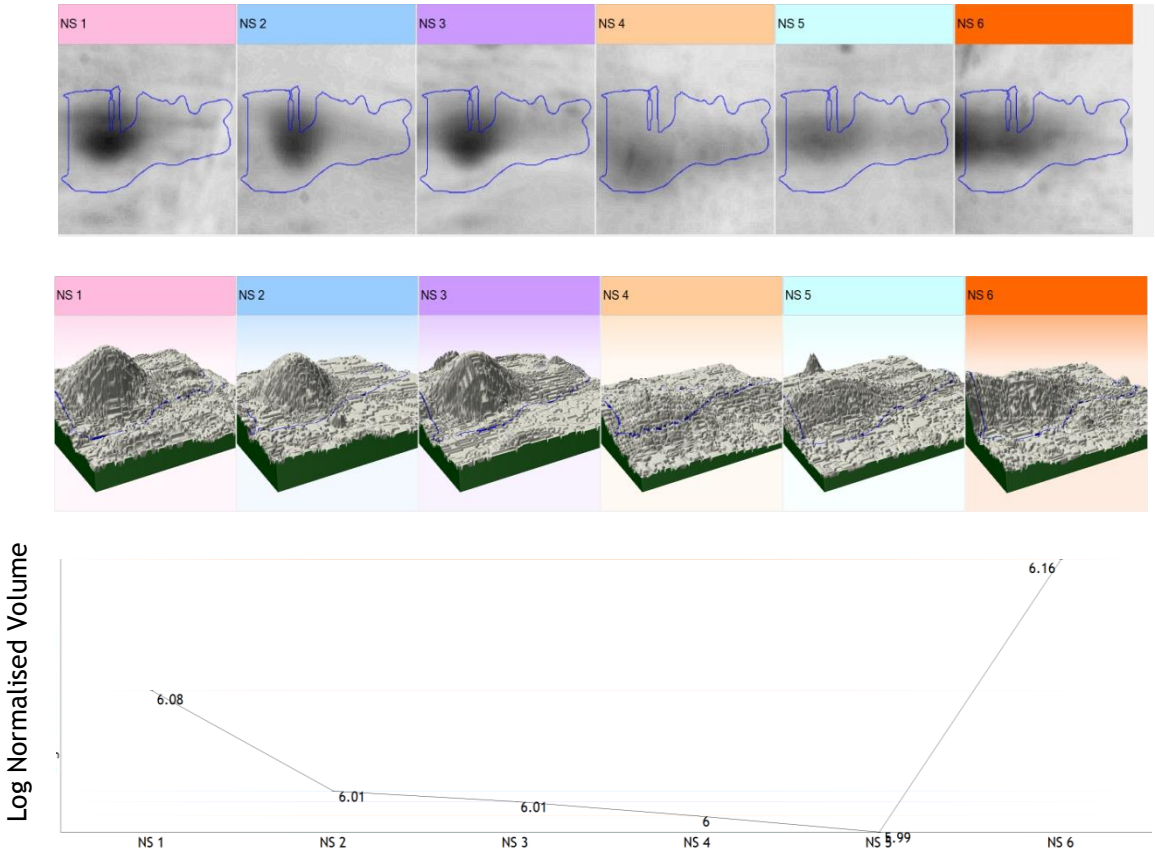


Figure 5.13: Variation in glutathione S-transferase spot intensity between individual NS samples. The median of glutathione S-transferase spot intensity between 6 different NS is 6.01 ranging from 5.99 – 6.16.

Odorant binding protein

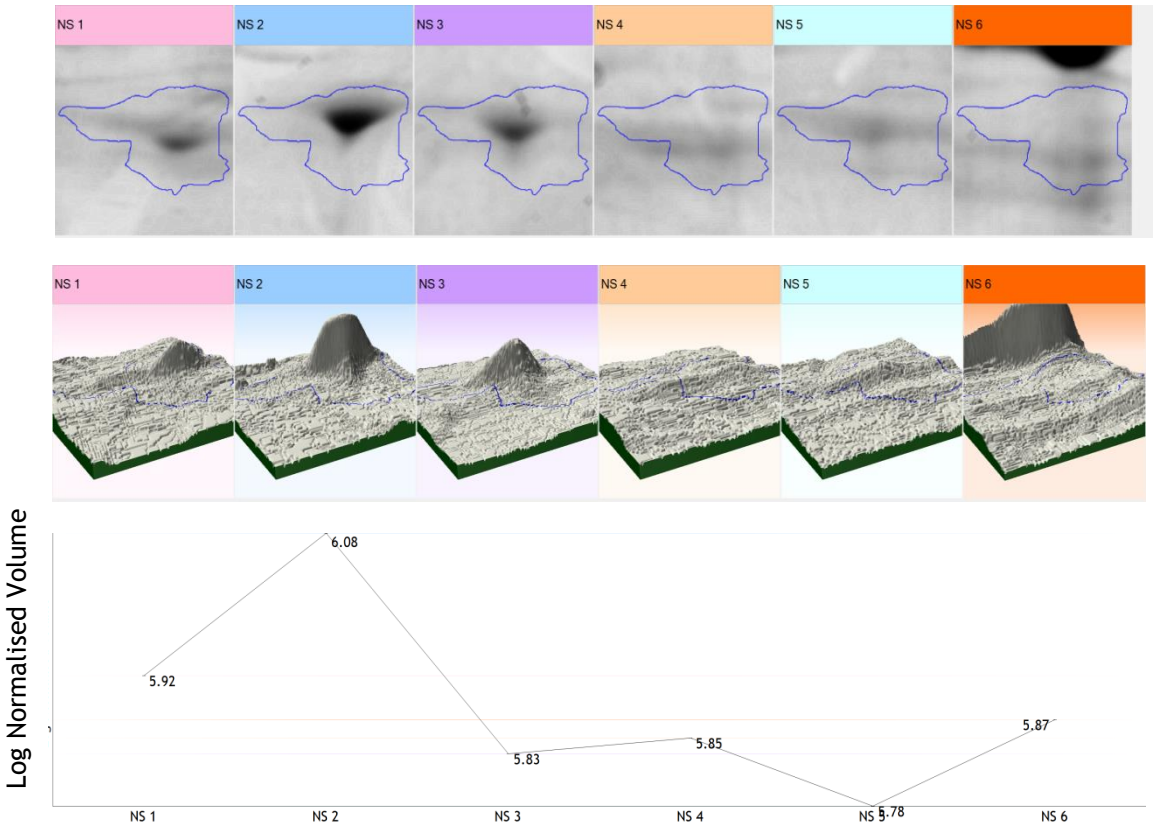


Figure 5.14: Variation in odorant binding protein spot intensity between individual NS samples. The median of odorant binding protein spot intensity between 6 different NS is 5.86 ranging from 5.78 – 6.08.

Immunoglobulin heavy chain

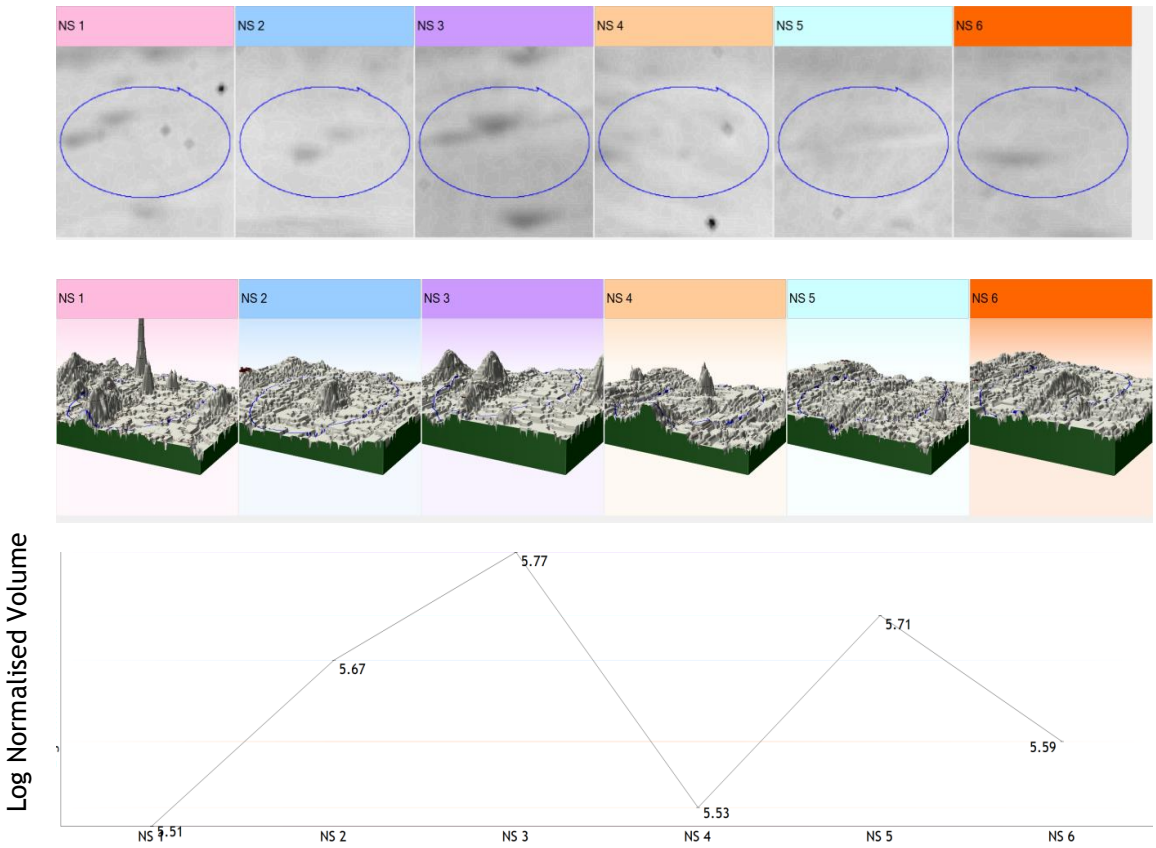


Figure 5.15: Variation in Ig heavy chain spot intensity between individual NS samples. The median of Ig heavy chain protein spot intensity between 6 different NS is 5.63 ranging from 5.51 – 5.77.

Immunoglobulin light chain

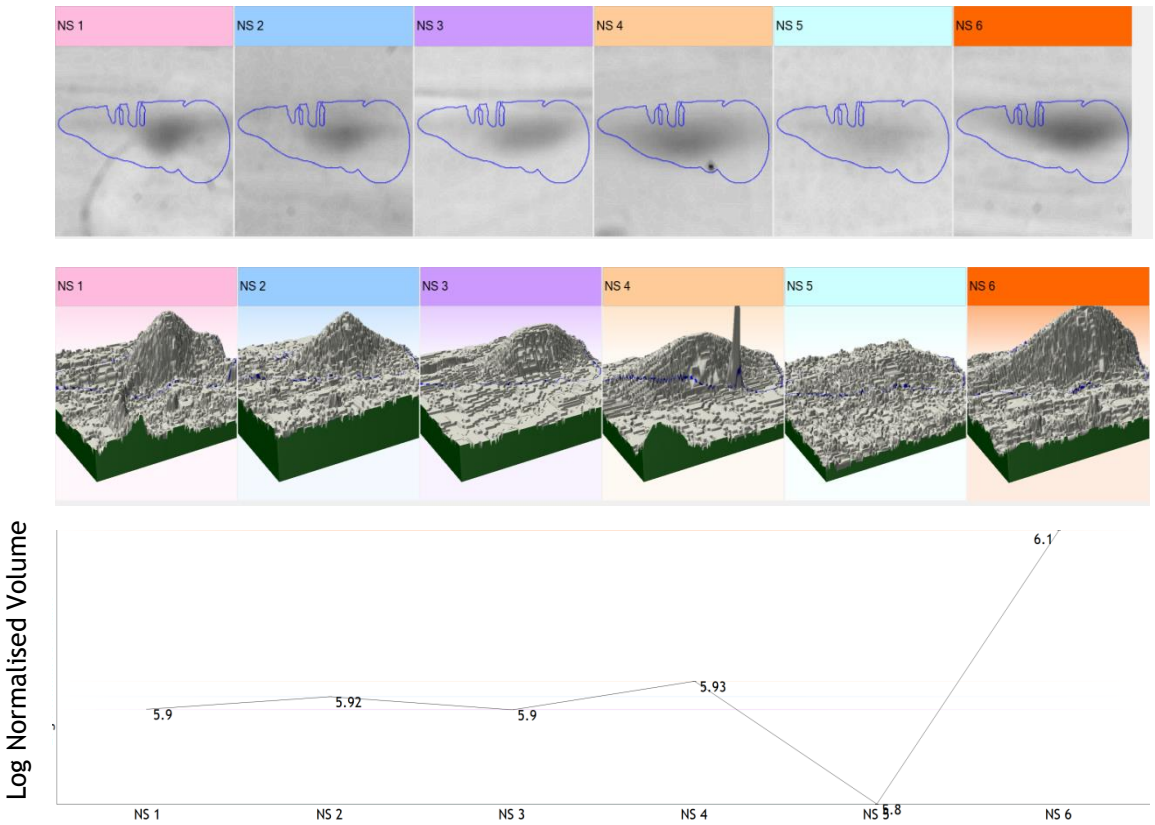


Figure 5.16: Variation in Ig light chain spot intensity between individual NS samples. The median of Ig light chain protein spot intensity between 6 different NS is 5.91 ranging from 5.8 – 6.1.

Serotransferrin

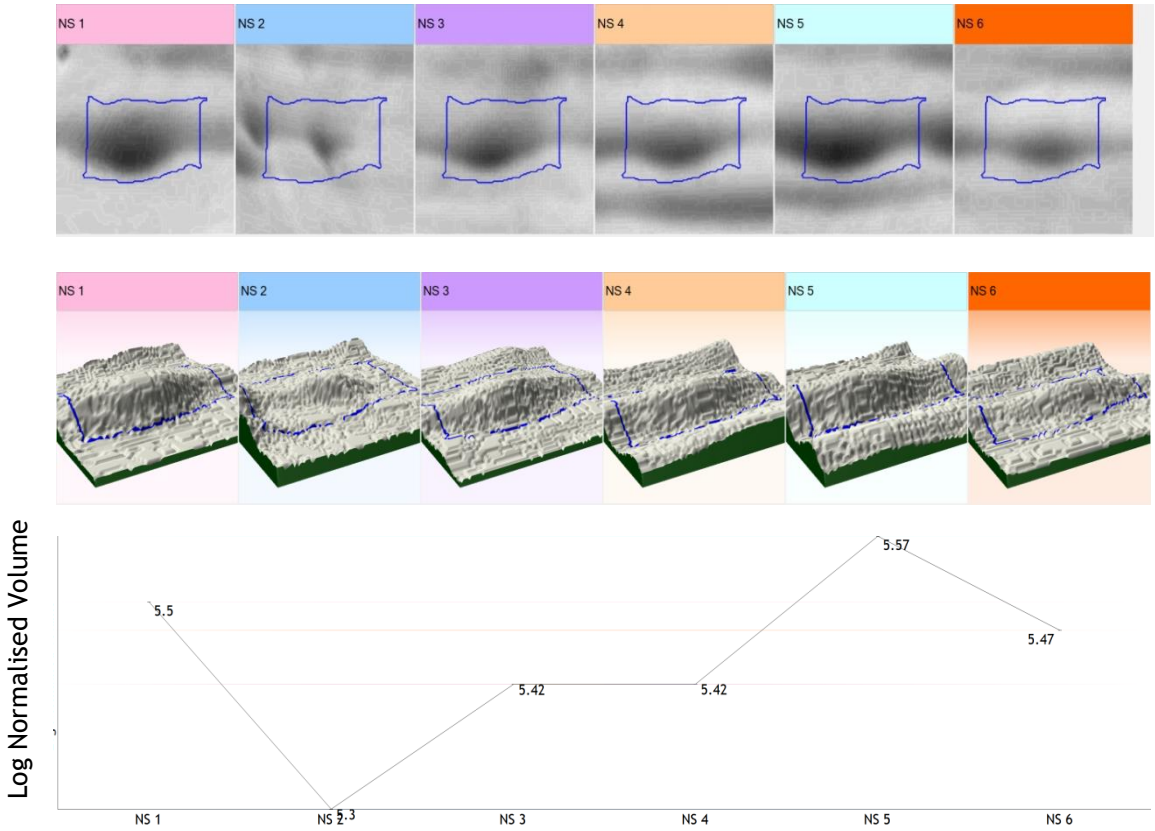


Figure 5.17: Variation in serotransferrin spot intensity between individual NS samples. The median of serotransferrin protein spot intensity between 6 different NS is 5.45 ranging from 5.3 – 5.57.

Lactoferrin

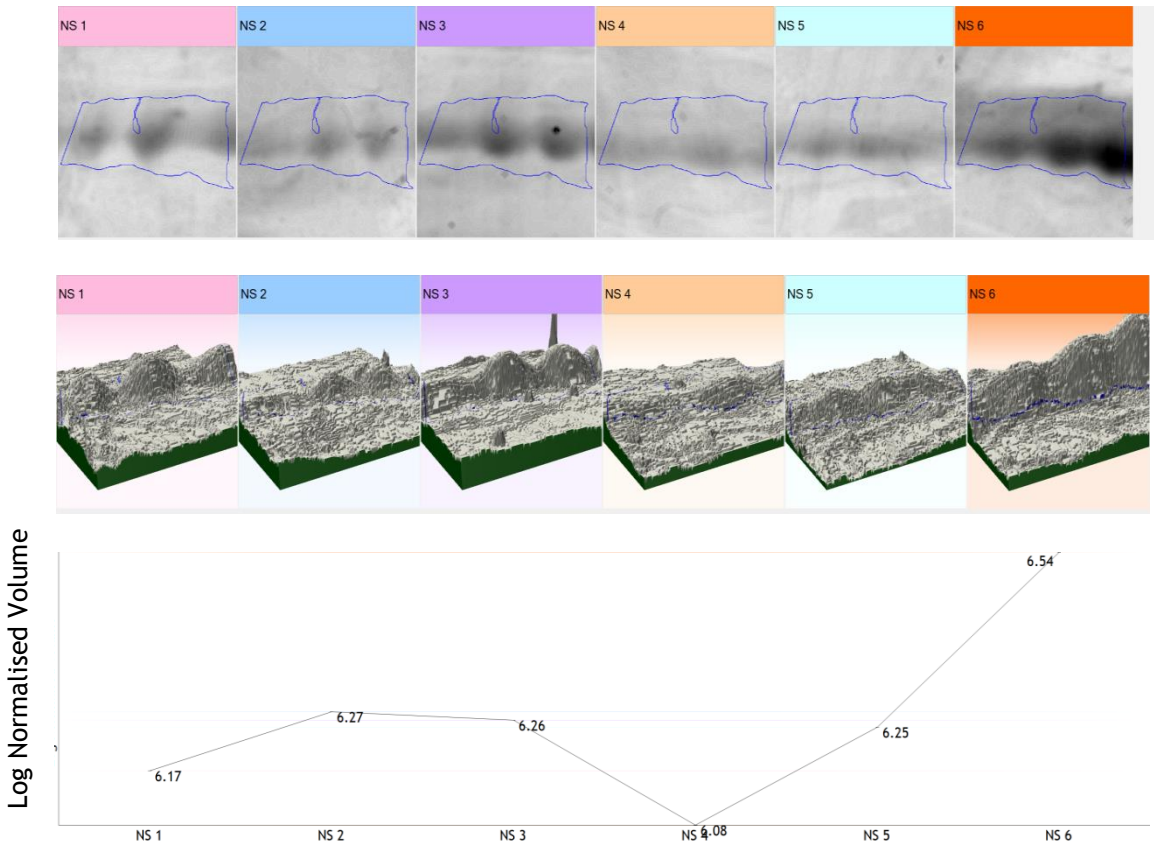


Figure 5.18: Variation in lactoferrin spot intensity between individual NS samples. The mean of lactoferrin protein spot intensity between 6 different NS is 6.26 ranging from 6.08 – 6.54.

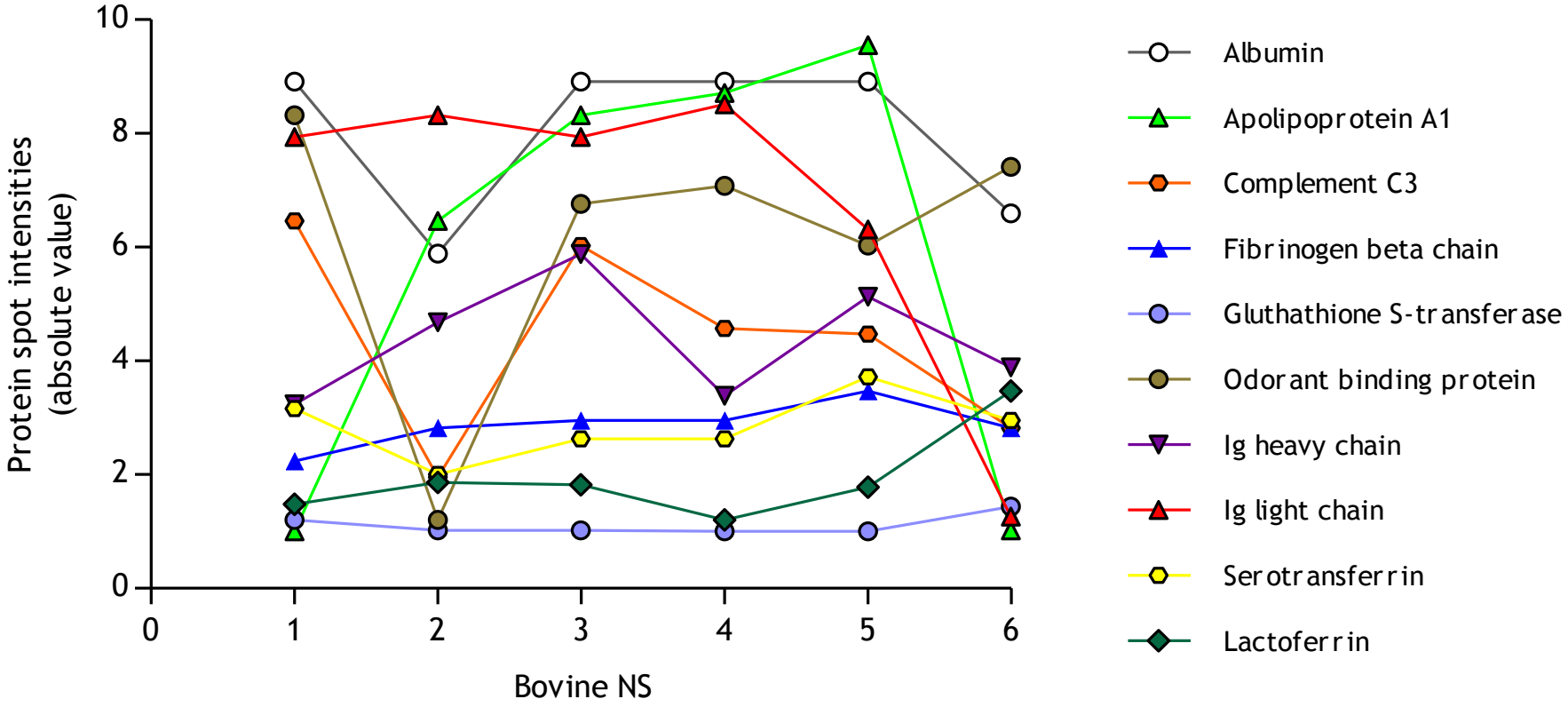


Figure 5.19: Measurement of the 10 major protein spot intensities in 2-DE gels from NS across 6 different healthy cattle.
Spot intensity results were converted from log normalise value to actual value of data.

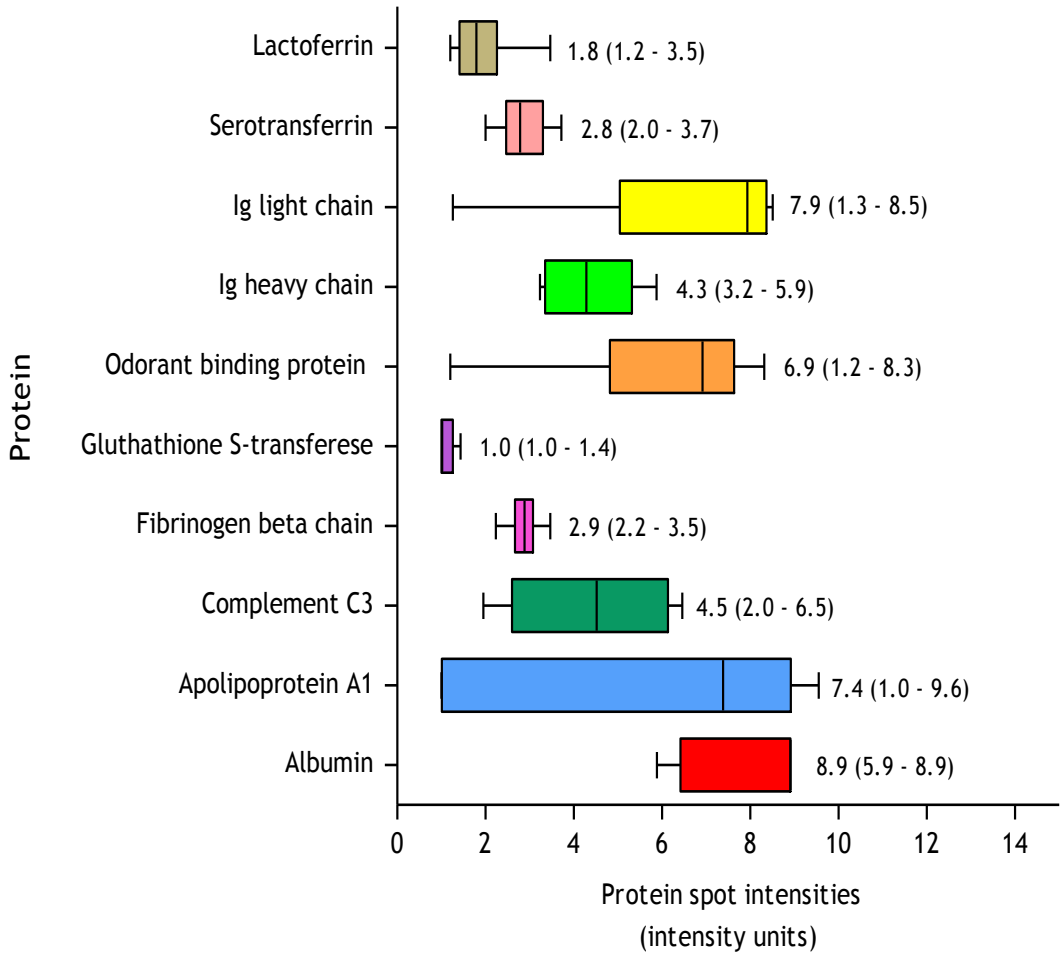


Figure 5.20: All major protein spot intensities from 6 2-DE gels were quantified and result presented in of median (range).

5.4 Discussion

The largest body surface in human and animal is the lining of the respiratory tract, gastrointestinal tract, and reproductive system each of which is covered by mucous membranes, named for their capacity to secrete mucus (Kaliner et al., 1984; Kaliner et al., 1986 and Wallace, 2008). This mucus forms a physical and chemical barrier as the first line of host defence against inhaled microbial invaders. Many studies of NS and mucus secretions in human have defined some of the physiologic and pharmacologic controls of human to maintain homeostasis and healthy (Kaliner et al., 1984; Allen et al., 1993; Casado et al., 2005 and Miyake et al., 2006). However, the constituents that are found in NS and their roles in animal health and disease are still in the initial phases of exploration. Bovine NS provides one potential and convenient source of mucous membrane secretions for biochemical and proteomic analysis, although certain NS mucoproteins have shown not to be able to penetrate 2-DE gel due to its relatively large size (Ghazali et al., 2014). Nasal secretion includes a variety of proteins, which appear to serve important functions in host defence. Most of the antiphlogistic proteins are synthesized and secreted by serous cells in the sub mucosal glands, and it appears that the serous cell is the resident antimicrobial cell in mucous membranes (Cole et al., 2002 and Joo et al., 2004).

Proteomics has become a valuable and essential tool for researchers to study the changes in protein expression level in the animal host at the time of stress and disease conditions (Eckersall and Whitfield 2011; Eckersall et al., 2012 and Almeida et al., 2014). The identification of NS proteins established in this study will promote the understanding of respiratory diseases and their fundamental pathological processes. Nasal secretion may also serve as a useful model for the analysis of lower pulmonary secretions in preventing airway colonization and pulmonary infections. Over the years, many groups have applied proteomic approaches to analyse differences in the human NS proteome mostly involving allergic rhinitis (Tomazic et al., 2013 and Tomazic et al., 2014) and chronic rhinosinusitis (Upton et al., 2011) with healthy controls. Most of the proteins identified in human NS (Casado et al., 2005) were similar with the nasal proteins detected in cattle with many proteins involved in innate and acquired immunity (Figure 5.7).

In this study, it has been demonstrated that the use of gel based proteomics either with 1-DE and 2-DE and MS protein allowed identification to catalogue the protein profile in bovine NS. One-DE gels on NS samples were able to separate proteins according to their molecular weight and 13 distinct protein bands were found. Upon MS analysis, 10

different proteins with the highest MOWSE score in each band but with 3 bands having a common protein, namely bovine serum albumin. However, apart from the proteins having the highest hits, MS results were also able to identify numerous other proteins within each band having MOWSE score of >100 and peptide matches of >3 (Figure 5.2). Therefore it was justified to apply 2-DE separation method to increase the separation of the proteins by pI as well as by Mw.

Two-DE was carried on NS from six healthy animals. The gel image revealed protein spots that were well distributed within the gel having protein spots at different pI and Mw. The initial 2-DE study demonstrated that there was a reasonable consistency between samples (see below) and following a 2-DE run with pooled samples the major protein spots visualized were sent for MS analysis. Most protein identified were similar to those found by 1-DE though glutathione S-transferase (GST) was also identified at around 25 kDa and at a basic form with pI values between 8.5 and 10.0.

The bovine NS contained high-abundant plasma proteins, such as albumin and immunoglobulins, glandular serous cell proteins (lactoferrin, OBP and polymeric immunoglobulin receptor) and epithelial keratins. Odorant binding protein has been demonstrated to have sequence similarity with a superfamily of soluble carrier proteins called lipocalins which include some proteins thought to be involved in the mechanism of releasing and modulating chemical messages with pheromonal activity (Pelosi, 1994) and also having actively involves in antimicrobial processes (Flower, 1996). Serous cells are also involved in forming adaptive immunity protection by transporting locally synthesized IgA. Mucosal plasma cells secrete IgA as a dimer link by the joining chain (IgJ). This secretory IgA is endocytosed and transported through the serous cells to be exocytosed into the gland duct mucus with other serous granule proteins.

Complement C3 has been widely believed to be important for bacterial inactivation and immune complex formation by activation of the classic complement cascade. Lactoferrin is presumed to be a part of the innate immune system and due to its strategic position on the mucosal surface lactoferrin represents one of the first defence systems against microbial agents invading the organism mostly via mucosal tissues. This protein has been reported to disrupt the growth and proliferation of a variety of infectious agents including both Gram-positive and negative bacteria, viruses, protozoa, or fungi (Kirkpatrick et al., 1971). Its ability to bind free iron, which is one of the elements essential for the growth of bacteria, is the key for the bacteriostatic effect of lactoferrin (Arnold et al., 1980). Brock (1980) has demonstrated that a lack of iron inhibits the growth of iron-dependent bacteria such as *E. coli*. Furthermore the

presence of fibrinogen highly suggests that these proteins participate with innate immune processes by forming coagulated mass of mucins, albumin and other components (Cassado et al., 2005).

Nasal secretion contains antimicrobial proteins and peptides as a first-line host defence against microbial invaders. Lactoferrin, stored in and secreted from serous cells in nasal submucosal glands, is the most abundant antimicrobial protein of NS but is also secreted from neutrophils in inflamed secretions (Niehaus et al., 2000). It is bactericidal for gram-positive bacteria and in combination for some gram-negative bacteria (Elisson and Giehl, 1991 and Raphael et al., 1989). Given lactoferrin's strong antibacterial properties it was suggested that together with AP both proteins constitutes the two major antibacterial proteins that were present and locally produced in NS. Other factors, or cofactors acting with lysozyme and lactoferrin, were also recorded to contribute to the antimicrobial properties of nasal fluid (Cole et al., 1999). It may be possible in the future to add AP to the list of endogenous NS anti-bacterial mediators as discussed in Chapter 3.

Bovine GSTs are soluble proteins with typical molecular masses of around 25-30 kDa, each composed of two polypeptide subunits. The GST proteins have evolved by gene duplication to perform a range of functional roles. Glutathione S-transferases also have non-catalytic roles, binding flavonoid natural products in the cytosol prior to their deposition in the vacuole (Alfenito et al., 1998 and Boušová et al., 2011). Recent studies have also implicated GST as components of ultraviolet-inducible cell signalling pathways and as potential regulators of apoptosis (Ishii et al., 2003). Glutathione S-transferases constitute the family of enzymes best known in their role in enzymatic detoxification of endo- and xenobiotics. In this capacity, the present of GST in NS may be to catalyse the conjugation of a number of electrophilic xenobiotic compounds to the tripeptide glutathione and in so doing make them less harmful. Glutathione S-transferases also provide protection against oxidative stress, catalyse various metabolic reactions and function as non-enzymatic carrier proteins for several ligands (Marrs, 1996 and Sheehan et al., 2001)

Apolipoprotein A1 is the major protein of plasma high density lipoprotein and plays an important role in lipid transport and metabolism. Apolipoproteins function in lipid transport as structural components of lipoprotein particles, cofactors for enzymes and ligands for cell-surface receptors. In particular, apolipoprotein AI is the major protein component of high-density lipoproteins. Additionally, apolipoprotein AI is known to have anti-inflammatory effects in rabbit by inhibits vascular inflammation in NZW rabbits

(Patel et al., 2010), negatively regulates ovalbumin-induced neutrophilic airway inflammation in mice (Dai et al., 2012) reducing the inflammatory response of sinusitis in human (Do Carmo et al., 2008).

The variability of the proteins between samples presented on graphs in Figure 5.19 and 5.20. Most of the nasal proteins are not normally distributed as shown by median and range but this may be due to some protein that is present in low abundance such as OBP, complement C3 and albumin seen in NS sample no. 2 and 6. The variations observed may be indicative of either individual differences or due to physiology or pathophysiology. Hence more work is needed to further investigate each of the individual protein present and determine their specific biological function in the NS.

In this chapter, it has successfully been demonstrated that proteomic separation technique such as 1-DE and 2-DE accompanied with ESI-MS/MS approaches can be used for the investigation and identification of NS proteins in animals. The main advantage of this method is the easier comparison across two or more sets of samples using imaging analysis. Although the 2-DE separation of the bovine NS have been successfully presented in this chapter particularly in protein expression and identification, a series of limitation have to be dealt with when using this approach. Determination of unambiguous protein identification in NS is difficult for the differential of protein expression between individual animals. The preparation of 2-DE needs intensive work and sample losses are frequent during electrophoretic separation because very basic and acidic proteins cannot often be detected. The considerable length of time for analysis and the issue of low reproducibility remain the major setback for using this method of separation. Not all proteins can be resolved, for example membrane proteins may not be well represented due to their poor solubility (Gygi et al., 2000 and Santoni et al., 2000). Similarly low abundant proteins, for example AP (Chapter 3) are not likely to be potential mucosal biomarkers using 1-DE or 2-DE because they are often lost during the electrophoretic procedure.

Recently, the concept of differential gel electrophoresis (DiGE) has been introduced to reduce gel-to-gel variability. Briefly, each of two samples is differentially labelled with fluorescence dyes (Cy3 and Cy5), and the two samples are then resolved simultaneously within the same 2D gel. Another important advantage of DiGE is that an internal standard labelled with the third dye commonly Cy 2 can be incorporated into the same gel, hence making the quantitative intensity analysis more accurate. The comparison of two samples has been reported to be satisfactory with DiGE (Wu, 2006). This new method will be discussed in the next chapter where the use of proteomics to compare

and differentiate the proteins present in NS of healthy, diseased and vaccinated cattle is discussed. Until recently, 2-DE and DiGE have remained the laboratory methods of choice to investigate the proteome, understanding the molecular changes or to perform comparative analysis of proteins.

Nevertheless, the use of gel based proteomics in this chapter as discussed earlier is not without disadvantages. However as describe in section 1.4, most of the drawbacks in gel electrophoresis could be possibly overcome by the alternative application of gel free proteomic method. The use of gel free proteomic method such as Multidimensional Protein Identification Technology (MudPIT) coupled with tandem MS/MS for protein identification has several important advantages. For example, MudPIT separates peptides in 2D liquid chromatography. This allows greater separation of peptides that can be directly interfaced with the ion source of a mass spectrometer, which maximizes sensitivity (Weerapana et al., 2007). This modern proteomic technology avoids the band broadening associated with many chromatographic steps, which can decrease resolution (Kislinger et al., 2005). The application of this procedure, however, also requires the manipulation of specimens with high purity to avoid contamination problems (Kislinger et al., 2005 and Abdallah et al., 2012).

Chapter 6

Comparative proteome analysis of nasal secretion from naive, malignant catarrhal fever challenged and vaccinated cattle

6.1 Introduction

6.1.1 Malignant catarrhal fever

Malignant catarrhal fever (MCF) is a generalized often fatal disease particularly in cattle, but also other ruminants including captive and farmed species of deer, antelope, water buffalo (Dettwiller et al., 2011), bison (Schultheiss et al. 1998 and Berezowski et al. 2005), and occasionally of domesticated pigs (Alcaraz et al., 2009 and Gauger et al., 2010). Malignant catarrhal fever is caused by gamma-herpesviruses of the genus macavirus, including alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2) (Russell et al., 2009 and Davison et al., 2009). These viruses infect their reservoir hosts (wildebeest for AlHV-1 and sheep for OvHV-2) efficiently without showing apparent clinical sign of disease but cause lymphoproliferative disease that is generally fatal when they infect susceptible hosts such as cattle, bison and deer. Malignant catarrhal fever is found worldwide wherever these susceptible hosts mix with reservoir animals (Russell et al, 2009).

The blue wildebeest (*Connochaetes taurinus*) is the major reservoir host, but black wildebeest (*Connochaetes gnou*) are also carriers. All or most wildebeest appear to be infected by this virus (Hamblin and Hedger, 1984). Domesticated sheep (*Ovis aries*) are the reservoir hosts for OvHV-2, and most individuals are infected. Zarnke et al. (2002) and Cooley et al. (2008) have reported some species of wild sheep, such as Dall's sheep (*Ovis dalli*) and mouflon (*Ovis musimon*) were also carriers. Goats are the carriers for CpHV-2 (Li et al., 2003). Most goats are thought to be infected with this virus, based on high seroprevalence to MCF viruses (Yeşlbağ, 2007); however goats can also be infected asymptomatically with OvHV2, and serological tests cannot distinguish these two organisms (Powers et al., 2005). Alcelaphine herpesvirus-2 (AlHV-2) is carried subclinically in hartebeest (*Alcelaphus buselaphus*) and topi (*Damaliscus korrigum*).

Up to the present, MCF still causes major economic losses to the cattle production worldwide especially for the African small-holders and local cattle industry (Russell et al., 2009). The AlHV-1 and OvHV-2 genomes have been completely sequenced, revealing that they are highly-related viruses (Ensser et al., 1997 and Hart et al., 2007). Malignant catarrhal fever virus is transmitted by contact or aerosol, mainly from wildebeest calves (AlHV-1) and lambs (OvHV-2) under 1-year old (Mushi et al., 1981 and Li et al., 1998). Incubation periods after experimental inoculation of cattle are 2–12 weeks (Buxton et al., 1984 and Taus et al., 2006). The causal viruses are passed between individuals of the reservoir hosts and from reservoir to MCF-susceptible species by the horizontal

route, although Plowright (1965) and Rossiter (1981) have reported that vertical transmission has been inferred from recovery of AlHV-1 from a wildebeest fetus and from the detection of anti-MCF virus antibodies in the serum of some gnotobiotic lambs respectively. In addition, Mushi et al. (1981) works on the isolation of AlHV-1 have shown that the principal source of free virus in wildebeest is in the tears and nasal secretions.

Several distinct patterns of clinical disease have been described for MCF in cattle: peracute, head and eye, alimentary, neurological and cutaneous (OIE, 2004). The head and eye form is the most common expression of disease in cattle. Common signs include pyrexia, inappetence, ocular and nasal discharge, lesions of the buccal cavity and muzzle, diarrhoea and depression. The clinical signs depend to some extent on the species infected, the virus and how long the animal survives after the onset of clinical signs. Many deer die within 48 hr of the first clinical signs and affected bison generally die within 3 days (O'Toole et al., 2002). In contrast, mortality seen in cattle can occur within a few days or weeks after the first clinical signs (Russell et al., 2012).

While a diagnosis on clinical and gross post mortem examination may be possible in acute and more prolonged cases with a typical signs of the disease, wide spectrum of clinical manifestations that can develop requires laboratory examination to arrive a definitive diagnosis. Characteristic lesions of this disease include histological changes of non-purulent vasculitis and interstitial infiltration of lymphoid cells in most of the tissues particularly the brain, kidneys and liver, which associated with the presence of necrotic ulcers at epithelial surfaces often provide the basis for reaching a diagnosis (Liggitt and DeMartini, 1980).

Laboratory diagnosis in MCF-susceptible species has benefited from recent developments in molecular virology. Sequencing of the genome of AlHV-1 has allowed the development of both generic and specific reagents for amplification of diagnostic fragments of both AlHV-1 and OvHV-2 genomes by PCR (Bridgen and Reid, 1991; Baxter et al., 1993 and Flach et al., 2002). A direct ELISA has also been developed recently that offers a simple and inexpensive alternative to other serological tests (Fraser et al., 2006). The use of PCR allows sensitive confirmation of the presence of MCF viruses in infected animals and may also be useful for phylogenetic and epidemiological studies in both natural and MCF-susceptible hosts (Russel et al., 2009). Conventional and real-time (quantitative) PCR assays have been developed for the detection of OvHV-2 and AlHV-1 viral DNA (Baxter et al., 1993; Flach et al., 2002 and Traul et al., 2005).

Additionally, the detection of MCF virus-specific antibodies or DNA in an animal with clinical signs will also support a diagnosis of MCF cases.

The current control that is successfully reducing the occurrence of clinical cases is only by minimizing the contact between carrier animals and susceptible livestock. Recently, an immunisation strategy developed by Haig et al. (2008) using prime vaccination and boost immunisations of attenuated AlHV-1 in adjuvant intramuscularly in the upper neck to stimulate a mucosal barrier of virus–neutralising antibody in the nasal and oro-pharyngeal region. This approach has successfully protected cattle challenged intranasally with a fatal dose of virulent AlHV-1. In addition, the same group also have conducted a proteomic study analysing the changes of peptides composition in AlHV-1 during the transition from virulent to attenuated virus in tissue culture (Dry et al., 2008). They have suggested that the different protein expression present between the virulent and attenuation of AlHV-1 were due to altered viral gene expression in the infected cell.

Duncan et al. (1972) and Morgan et al. (1981) have reported the normal values of IgA and IgG concentration in bovine nasal secretion were ranged between 1.8 - 3.9 and 0.1 - 0.7 g/L respectively. Both of the mucosal antibodies have been reported to increase in nasal secretion in vaccinated and experimentally challenged cattle against *P. haemolytica*, IBR and PI-3 viruses (Mukur et al., 1975; Komar et al., 1975 and Nelson and Frank, 1989). As reported in Chapter 2, bovine NSAP activity from apparently healthy cattle (n=38) have been shown to be 15.7-fold higher at 80:1259 IU/L than the serum reference range for AP. Subsequently, the bio and genomic characteristic and possible physiological function of bovine NSAP have been discussed in detail in Chapter 3 and 4. The NSAP activity during MCF and vaccination has not been measured or reported. Hence this could provide a novel approach to study and better understand the progression of host response towards diseases and vaccinations.

Likewise, currently there is no known proteomic study that has analysed the changes in protein composition of NS from animal infected by AlHV-1, or indeed any bovine respiratory disease. As mentioned above the most common route of MCF virus transmission reported is by aerosol and NS being the primary innate defence mechanism against the causative agent, it is possible that changes in the NS protein composition occur in animals infected with MCF. Therefore, this chapter describes a novel use of difference gel electrophoresis (DiGE), a modern comparative proteomic technology, to analyse the NS proteins in animal infected and vaccinated against AlHV-1.

6.1.2 Difference gel electrophoresis

In 1997, Unlu and co-workers first described the 2-D Difference gel electrophoresis (DiGE) technique to profile proteins (Unlu et al., 1997). The method of DiGE overcomes the reproducibility problems of conventional 2-DE by labelling proteins with spectrally distinct fluorescent dyes prior to electrophoretic separation with different samples run on a single gel. The dyes used are cyanine dyes (GE Healthcare DiGE CyDye Fluorescent CyTM, Hatfield) and there are three spectrally distinct CyDyes available: Cy2, Cy3, and Cy5. These are used to label different samples allowing up to three samples bound to the three different CyDyes to be electrophoresed on the same gel. The gel is then scanned with a fluorescent imager at different wavelengths and each of the samples on the single gel is visible separately. All three CyDye DiGE fluorescent minimal dyes have different absorption and emission spectra. Because of the different absorption and emission spectra, the CyDyes can be used for multiplexing of up to three different protein samples on the same 2-DE gel. Due to the high degree of sensitivity the labelling method used, a single protein as low as 125pg can be detected following electrophoresis (Chakravarti et al., 2005).

There are two types of DiGE labelling chemistries available, one labels lysine residues within sample while the other labels cysteine, these are termed minimal and saturation labelling respectively. Lysine is the most widely employed labelling method due to the reactive *N*-hydroxysuccinimide ester group that forms a covalent bond with the *ε*-amino group of lysine side chains. The content of lysine is generally very high in most proteins such that an excessive amount of dye is required to direct the reaction to completion (Westermeier et al., 2008). Labelling of the lysine with the CyDyes is extremely sensitive, with picogram sensitivities (Chakravarti et al., 2005). Minimal labelling CyDyes are supplied as N-hydroxy Succinimidyl esters, which react with primary amino groups, typically the terminal amino group of lysine side chains; with labelling reactions optimised so only 2.5% of the total number of lysine residues is labelled (Lilley & Friedman, 2004). If all of the lysines were labelled the proteins would become too hydrophobic and not stay in the solution (Westermeier et al., 2008).

Whereas in a minimally labelled DiGE experiment samples that are to be compared are labelled with either Cy3 or Cy5 CyDye fluorescents (Lilley & Friedman, 2004). For samples to be compared quantitatively and the comparison to confer any statistical power then a pooled internal standard must be utilised and ran on all gels (Unlu et al., 1997 and Chakravarti et al., 2005). The pooled internal standard comprises equal aliquots of every sample within the experiment and is labelled with the Cy2 CyDye. This

will result in three differentially labelled samples: two samples labelled separately to Cy3 and Cy5 and the pooled internal standard labelled with Cy2. These are then mixed and co-resolved in the same 2-D gel (Kondo and Hirohashi, 2006). The use of the pooled internal standard could ensures that all proteins in the sample are represented assisting both inter and intra gel matching.

The DiGE technology is able to prevent the issue of variation in spot volumes due to gel-specific variation such as sample entry and electrophoresis in either the first dimension: immobilised pH gradient (IPG) strip or second dimension: SDS-PAGE. Having been ran together under identical condition with proteins migrating under the exact conditions the problems of reproducibility are overcome (Unlu et al., 1997; Lilley and Friedman, 2004 and Westermeier et al. 2008). Furthermore the relative amount of a protein in a gel in one sample compared with another will be unaffected (Lilley & Friedman, 2004). Additionally, labelling with CyDyes have shown to allow greater level of detection of protein spots than Coomassie Blue and a comparable level of detection of protein spots as with silver stain or SYPRO Ruby (Gharbi et al., 2002 and Gade et al., 2003).

For quantification, measurements are made relative to the Cy2 signal for each resolved protein (Chakravarti et al., 2005). The Cy2 signal should be the same for a given protein across all the different gels because it came from the same bulk mixture, therefore any differences represent gel-to-gel variation, which can be effectively neutralised by normalising all the Cy2 values for a given protein across all the gels (Friedman and Lilley, 2008). Therefore, by using the Cy2 signal to normalise the ratios between the DiGE gels could provide direct comparisons of spot volumes to be made between the Cy3 and Cy5 labelled samples and the Cy2 labelled internal standard for that gel (Tonge et al., 2001 and Lilley and Friedman, 2004).

The use of sufficient replicates and the presence of the same pooled standard present in each gel allows for the application of the students t-test and analysis of variance (ANOVA) statistical analysis despite the samples having been separated on different DiGE gels (Lilley and Friedman, 2004). The imaging software developed for the DiGE system: DeCyderTM (GE Healthcare, Hatfield) is typically used for analysis. DeCyder is able to process all the scanned gel images, detect the spots, match spots across the gels and perform statistical analysis. The DeCyder software package has a triple codetection algorithm that allows simultaneous detection of labelled protein spots from images that arise from the same gel and increases the accuracy in the quantification of standardised abundance. The standardised abundances can then be compared across groups to detect changes in the protein expression (Alban et al., 2003).

The minimal labelling method in DiGE technology is compatible with the downstream processing of selected protein spots. Spots, usually selected based on their differential expression between experimental groups are excised from the gels and trypsin digested to yielding tryptic peptides of an appropriate size efficient for mass spectrometry analysis. Mass spectrometry is an analytical technique that measures the mass-to-charge ratio (m/z) on ions based on their motion or magnetic field (Westermeier et al., 2008). For protein identification selected protein spots are firstly trypsin digested, which cleaves peptides bonds at the carboxylterminal of lysine and arginine amino acid residues (Lilley and Friedman, 2004 and Chakravarti et al., 2005). The peptide masses are determined using the mass spectrometer to create a unique peptide mass fingerprint that makes it possible to infer the amino acid composition of those peptides. Subsequent identification of the protein is achieved by using software such as Mascot (Matrix Science) that assimilates mass spectrometry data and identifies proteins, matched from other genomic and proteomic database, such as NCBI and SWISS-PROT.

6.1.3 Aims of study

The aims of the work presented in this chapter were to:

4. Measure and compare the NSAP activity in pre and post-challenge animal with AlHV-1.

5. Measure and compare the nasal mucosal antibody, IgA and IgG concentration in pre and post-challenge animal with AlHV-1.

6. Determine the changes of protein expression in NS of healthy, MCF challenge and vaccinated cattle by DiGE technology.

6.2 Materials and methods

6.2.1 Animals

Samples of bovine NS were obtained from cattle involved in MCF immunisation studies carried out at the Moredun Research Institute (MRI), Scotland and were provided by Dr G. Russell (Haig et al., 2008). Twenty three disease-free and OvHV-2 seronegative male Friesian-Holstein cross-calves between 3 and 5 months of age were used in the experiments. The animal experiments were carried out with the approval of the MRI's experiments and ethics committee and complied fully with the Home Office of Great Britain and Northern Ireland "Animals (Scientific Procedures) Act 1986". Animals exhibiting a rise in rectal temperature >40 °C for 2–4 days along with inappetance and ocular or nasal discharge were euthanized with an overdose of intravenous sodium pentobarbitone.

6.2.2 Immunisation study design

The calves were randomly assigned to 6 different groups (Table 6.1). On week 0, naive and disease calves were treated with sterile phosphate buffered saline (PBS). Vaccinated calves were treated by intramuscular (IM) at the upper neck region with either complete Freund's adjuvant (CFA) or attenuated C500 AlHV-1 in CFA [$10^{7.3}$ to $10^{7.5}$ $tcid_{50}$/ml virus, where $tcid_{50}$/ml is the tissue culture infective dose of virus giving a cytopathic effect (CPE) in 50% of wells containing cultured fibroblasts] as primary immunisation. Immunisation boosts were given 4 weeks later by IM or intranasal (IN) with either incomplete Freund's adjuvant (IFA) or attenuated AlHV-1 in IFA or adsorbed in chitosan [0.2% (w/v) in sodium acetate buffer pH 7.5].

On week 10, all of the calves were administered virulent C500 strain AlHV-1 as challenge either by intravascular (IV) at the dosage of $10^{4.5}$ $tcid_{50}$/ml virus or IN (10^{4} $tcid_{50}$/ml virus) route (Table 6.1). Calves that succumbed to MCF or showing clinical sign of MCF were euthanized and post mortem for examination of the mesenteric lymph nodes. Survived animals were euthanized 12 weeks after challenged to look for any signs of infection or disease.

Group (no. of animals)	Vaccine type	Primary Immunisation Route	Booster Type	Booster Route	Pre-challenge sampling	Challenged Route	Post-challenged sampling
		Week 0		Week 4	Week 8	Week 10	Week 12
1 (n=4)	Complete Freund's Adjuvant	IM	Incomplete Freund's Adjuvant	IM	Collection of NS	IV	Collection of NS
2 (n=5)	Att. AlHV-1 Virus		Att. AlHV-1 Virus	IN			
3 (n=3)	Att. AlHV-1 Virus		Att. AlHV-1 Virus	IM			
4 (n=4)	Att. AlHV-1 Virus		Att. AlHV-1 Virus	IN		IN	
5 (n=3)	Att. AlHV-1 Virus		Att. AlHV-1 Virus	IM			
6 (n=4)	PBS		PBS	IM			

Table 6.1: The experimental design details of the immunisation and control groups used in this study.
AlHV-1= Alcelaphine Herpesvirus-1; Att.=attenuated; PBS=phosphate buffer solution; IM=intramuscular; IN=intranasal; IV=intravenous.

6.2.3 Bovine nasal secretion

Nasal secretion samples were collected from the experimental animal at d-56 and at d-84 post AlHV-1 immunisation by Dr. G. Russell of MRI. The NS were processed as described in section 2.2.2. and were stored in -80°C until further analysis.

6.2.4 Alkaline phosphatase activity

Alkaline phosphatase activity in NS pre and post challenge was measured using pNPP enzymatic reaction as described in section 2.2.5.1.

6.2.5 Analysis of mucosal antibody response

Analysis of nasal mucosal antibody mainly IgA and IgG was performed by measuring their concentrations in NS during pre and post challenge using species-specific ELISAs (Bethyl Labs, USA) as described in section 2.2.6.

6.2.6 Difference gel electrophoresis

6.2.6.1 Experiment design

Three different CyDye DiGE Fluorescent minimal dyes are available for protein labelling: Cy3, Cy5 and Cy2, each of which can be visualised separately when scanned with a fluorescent imager at different wavelengths. The selections of NS for DiGE experiment were based on the post-challenge outcome result (section 6.3.1). Three groups of NS samples were used to represent the healthy, disease and vaccinated group (Table 6.2).

DiGE experimental group	Source of nasal secretion sample used (AlHV-1 immunisation group)*
Healthy	Group 6 pre-challenge
Diseased	Group 6 post-challenge
Vaccinated	Group 5 post challenge

Table 6.2: The different types of NS used for DiGE experiment and their designated group
*Nasal secretion samples from Table 6.1

Figure 6.1 summarized the experimental design for the DiGE experiment. Fifty µg of NS protein was pooled according to their respective group: healthy (n=4), disease (n=4) and vaccinated (n=3). Subsequently, the NS proteins were bound separately to both Cy3 and Cy5 fluorescent dyes according to their respective DiGE gel shown in Figure 6.1. Each gel contained a CyDye DiGE Fluorescent Cy2 dye labelled pooled internal standard, which containing equal volumes from control and challenged groups at each time point. Bound to Cy2 this acted as an internal standard throughout the DiGE experiment. For each group of animal two gels were ran, with the respective samples bound to opposite Cy dyes, together with the Cy2 internal standard. DiGE images were analysed using DeCyder v7.0 and protein spots that were found to be differentially expressed in the disease and vaccinated group identified. Spots were picked from preparatory gels, ran alongside the DiGE gels. A preparatory gel was ran for the pooled healthy, disease and vaccinated group and was loaded with a greater concentration of protein at 500 µg, making spot picking and identification of the protein possible. Both DiGE and preparatory gels were prepared and run by Mr. A. Scott of Glasgow Polyomics.

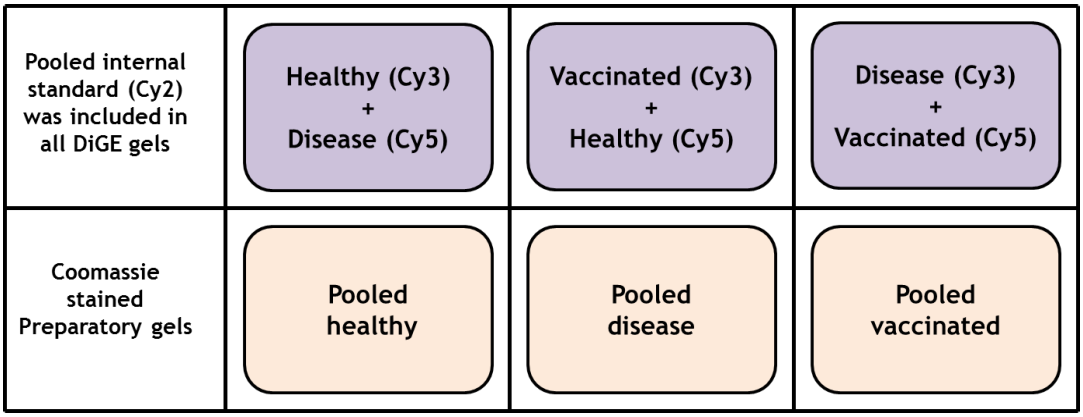


Figure 6.1: Experimental design for DiGE experiment (n=3) and Coomassie stained Preparatory gels (n=3) and layout of samples in gels.
Healthy=group 6 pre-challenge (n=4); Disease=group 6 post-challenge (n=4) and Vaccinated=group 5 post-challenge (n=3).

6.2.6.2 Preparatory gels

For each of the 3 different groups: pooled healthy (Group 6 pre-challenge), pooled diseased (Group 6 post-challenge) and pooled vaccinated (Group 5 post challenge) group, 500 µg of protein was mixed with 350 µl of rehydration buffer (6M urea, 2M thiourea, 4% CHAPS, 0.002% (w/v) bromophenol blue) into which 3.5 mg of dithiothreitol (DTT) and 1.75 µl IPG pH 4 - 7 buffer (GE healthcare biosciences 17-6000-86, Hatfield) had been added. These were mixed for 30 min and added into individual strip holders

("coffins") which hold the sample for focusing. A 24 cm pH 4-7 IPG strip (GE Healthcare Life Sciences 17-6002-32, Hatfield) was placed gel side down into the coffins. One ml of mineral oil was added over the IPG strip. Each IPG strip was focused to 8000 V over 27 hr.

Following focusing the IPG strips was washed in two IEF equilibrium buffer washes for 15 min each. The first with 10mg/ml DTT added and the second with 25mg/ml of 2-iodoacetamide added. Each IPG strip was placed between the two halves of the gel cassette directly onto the top of a 24cm 12.5% SDS-PAGE gel. One ml of 0.5% agarose with bromophenol blue was added on top of the gel to seal the strip to the gel and to serve as a dye front for monitoring the progress of the gel. The gel cassettes were placed into the ETTAN DALT system with 2% SDS running buffer. The gels were run at 1V per gel for 18 hr. The gels were fixed for 1 hr in 40% ethanol / 10% acetic acid, washed twice for 10 min in dH_2O and stained in colloidal Coomassie for 48 hr.

6.2.6.3 DiGE gels

Equal masses of total protein (0.5 mg) from healthy, diseased and vaccinated group were pooled separately. The pooled samples were then labelled with 400 pmol of different CyDye Fluorophors. Each DiGE gel had two samples of 50µg of protein bound to either Cy3 or Cy5 dyes together with 50µg of the Cy2 bound pooled internal standard. To bind the Cy dyes to the protein, the prepared samples were mixed with 1µl of Cy3 and Cy5 fluorescent dyes separately and placed in the dark on ice for 30 min. One µl of 10mM lysine was then added to stop the Cy's reaction with the protein. For each gel the Cy3 bound sample, the Cy5 bound sample and the Cy2 bound protein were mixed together for each gel as detailed in Figure 6.1, with rehydration buffer containing DTT and IPG buffer and added to a coffin with a 24cm 4-7 IPG strip (GE Healthcare Life Science 17-6002-32, Hatfield, UK). The strips were focused as described for the preparatory gels. Similarly, for the second dimension the strips were washed as described for the preparatory gels and ran at 1 V per gel for 18 hr.

6.2.6.4 Protein separation

The separation was carried out on IPGphor (GE Healthcare, Hatfield) in the first dimension (IEF electrophoresis, pI 4-7), followed by equilibration and reduction of IEF-strips according to standard protocols (Hannigan et al., 2007 and McNamara et al., 2011). Focused IPG strips were then loaded on top of SDS-polyacrylamide gels (13% total

acrylamide, 3% bis-acrylamide), and the 2-DE was carried out using a DALT 12 vertical gel electrophoresis system (GE Healthcare, Hatfield UK).

6.2.6.5 Image analysis of DiGE gels

After 2-DE, gels were scanned using Typhoon™ 9400 Imager (GE Healthcare, Hatfield), the protein spots were analysed with DeCyder 2-D Differential in-gel analysis (DIA) software v7.2 (GE Healthcare, Hatfield) and biological variation analysis (BVA) software. The BVA processes multiple gel images and also performs gel-gel matching of spots allowing quantitative comparisons of protein expression across multiple gels (GE Healthcare DeCyder User manual, Hatfield). A direct comparison between of spot volumes was made between the Cy3 and Cy5 labelled samples and the Cy2 labelled sample for that gel. The ratios were normalised and compare with those generated for that particular spot from other gels. The presence of the same pooled internal standard in each gel allowed for the application of the two-way analysis of variance (ANOVA) statistical analysis (Lilley and Friedman, 2004).

The statistical analysis in DeCyder is based on the standardized protein log abundances, which are defined as the log10 of the standardized abundances (Fodor, et al. 2005). The standardized abundance is derived from the normalized spot volume standardized against the intra-gel standard. The logs of the standardized abundance values are used in order that the data points approach a normal distribution around zero, thereby fulfilling the requirements of the subsequent statistical tests (GE Healthcare DeCyder User manual, Hatfield). In this study, one-way ANOVA was used to evaluate the protein expression of NS from the healthy, disease and vaccinated group of animals.

As this experiment assessed the variation of protein expression in NS in 3 different conditions of animal, a one-way ANOVA was used to identify protein spots that were differentially expressed as a result of the intranasal challenge of AlHV-1. The preparatory gel images were loaded into the BVA module, designated as pick spot maps, and matched to the DiGE gels. Differentially expressed protein spots whose log standardised abundance was statistically significant for 3 different conditions were selected for identification, matched to the pick spot maps and subsequently excised from the preparatory gel for tryptic in-gel digestion and protein identification.

6.2.7 Protein digestion and mass spectrometry analysis

Protein tryptic in-gel digestion was performed as described in section 5.2.8, while MS analysis and protein identification were carried out as described in section 5.2.9 and 5.2.10 respectively.

6.2.8 Statistical Analysis

Data for post-challenge survival percentage between groups were analysed by sample paired t-test between percent (www.statpac.com/statistics-calculator/percents.htm) to compare experimental groups. Nasal secretion AP, IgA and IgG response data of pre and post challenge were compared using paired t-test. DeCyder, after spot matching, one-way ANOVA statistical test was used to select protein spots which were differentially expressed between the healthy, disease and vaccinated group base on the BVA module. P value < 0.05 is considered significant for both tests.

6.3 Results

6.3.1 Post-challenge outcome

Table 6.3 shows that all the animals in group 5 (IM prime with attenuated virus in CFA and IM boost with attenuated virus in IFA) and 2 of 4 animals in group 4 (IM prime with attenuated virus in CFA and IN boost with attenuated virus adsorbed to chitosan adjuvant) survived a lethal challenge with AlHV-1 given by the intranasal route. Only the immunisation regime for group 5 produced significant protective effects (P = 0.04, group 5 vs. groups 6) whereas the immunisation regime for group 4 did not produce significant protective effects (P = 0.21, group 4 vs. groups 6).

Two of the group 5 animals were euthanized 12 weeks after challenge to look for signs of infection or disease. The remaining group 5 animals survived for a year after challenge, when it was euthanized to terminate the experiment. All of the control animals given PBS without immunising virus and challenged intranasally with virulent virus succumbed with MCF between 3 and 7 weeks after challenge.

Group 3 animals, given the same immunisation regime that vaccinated group 5 cattle against intranasal virus challenge, all succumbed with MCF when the virus was given systemically. These animals developed MCF over a similar time period as unimmunised control group animals given virus systemically and compared with group 1, no difference between the 2 groups as no animal survive the challenge.

Adjuvant alone had no MCF-protective effect as all the animals in group 1 died within the second week post-challenge. Finally, of the unvaccinated animals, those challenged intravenously with virus were seen to develop clinical signs of MCF earlier than those that were intranasally challenged. The animals exhibiting a rise in rectal temperature >40 °C for 2–4 days along with inappetance and ocular or nasal discharge (Figure 6.2) were euthanized with an overdose of intravenous sodium pentobarbitone.

Group (no. of animals)	Treatment Type	Treatment route (primary/booster)	Challenge route	Death 0-12 w post-challenge	Survivor > 12 w post-challenge
1 (n=4)	Adjuvant	IM/IM	IV	4	0
2 (n=5)	Att. virus	IM/IN		5	0
3 (n=3)	Att. virus	IM/IM		3	0
4 (n=4)	Att. virus	IM/IN	IN	2	2
5 (n=3)	Att. virus	IM/IM		0	3
6 (n=4)	PBS	IM/IM		4	0

Table 6.3: Post-challenge outcome of the immunisation procedures.
The number of animal showing onset of MCF clinical signs either died or euthanized within 12 weeks post challenge are indicated in the 'death' column while animals that survived for autopsy at the end of the experiment are indicated on the 'survivors' column (*). Att=attenuated; PBS=phosphate buffer solution; IM=intramuscular; IN=intranasal; IV=intravenous.

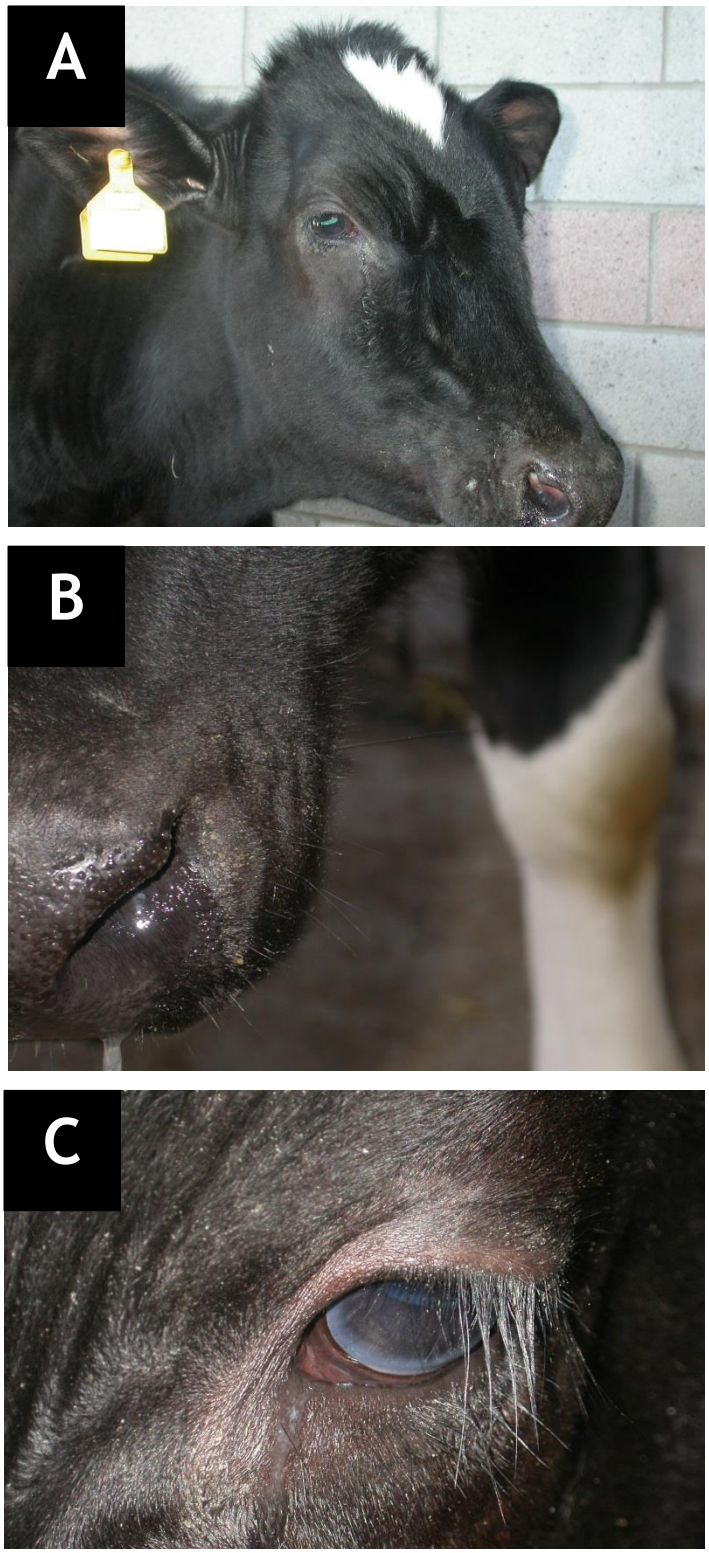


Figure 6.2: Clinical signs of MCF seen in cattle after challenge with AlHV-1 were euthanized. (A) Cattle showing clinical signs of increase in nasal and ocular discharges. Close up images of nostril (B) and eye (C) shows increased in nasal and ocular secretion respectively. (Images courtesy of Dr. G. Russell)

6.3.2 Response of nasal secretion alkaline phosphatase activity on AlHV-1 challenge

Following intranasal challenge with AlHV-1, the NSAP activity was measured pre and post-challenge in the animals that have been vaccinated (group 4 and 5) and those that not receive any kind of vaccination (group 6) to observe whether the immunisation regime and MCF disease have an effect on the high AP activity seen in NS of healthy animals (Chapter 2). The result data showing the changes of NSAP activity on day 14 after receiving AlHV-1 challenge are shown in detail in Figure 6.3.

There was no significant change in NSAP on day 14 post challenge but the NSAP activity in both vaccinated groups tended to be higher than the pre-challenge NSAP activity however in the disease group, NSAP activity tended to decreased. Nevertheless, all the NSAP activity finding pre and post-challenge in all the 3 animal groups were similar with the AP activity finding in Chapter 2 where it showed a higher AP activity in the NS in compare to the serum reference.

Paired t-test comparison using GraphPad Prism v5, reveal that animal challenge with AlHV-1 have no effect on the NSAP activity in both vaccinated group 4 and 5 having no significant difference and mean±(SD) of ($p=0.26$, 3220±1290 IU/L) and ($p=0.06$, 1900±170 IU/L) respectively. Similar finding was also seen in the disease group 6 where the decreased NSAP activity observed was not significantly difference ($p=0.13$) having a mean±(SD) of 1608 ±1666 IU/L.

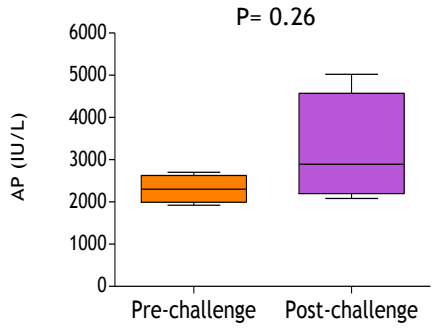
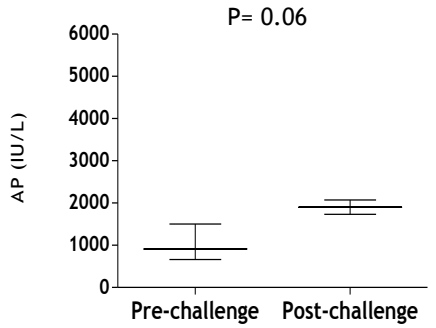
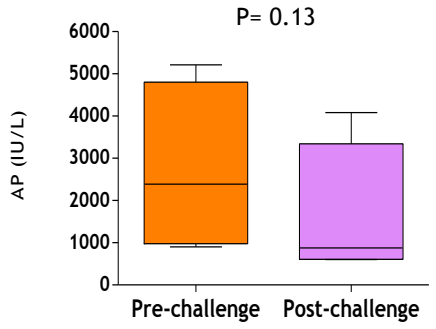
	Treatment type	Treatment route (primary/booster)	Challenge route	AP activity (IU/L)
Group 4 (n=4)	Att. virus	IM/IN	IN	
Group 5 (n=3)	Att. virus	IM/IM	IN	
Group 6 (n=4)	PBS	IM/IM	IN	

Figure 6.3: Nasal secretion AP activity (IU/L) response from cattle challenge intranasally with AlHV-1.
Data comparison of median NSAP activity pre and post-challenge by paired t-test. Att=attenuated; PBS=phosphate buffer solution; IM=intramuscular; IN=intranasal

6.3.3 Response of mucosal antibody in nasal secretion

Following intranasal challenge with AlHV-1, the IgA and IgG mean concentration in NS of group 4, 5 and 6 has shown to be significantly increased. The result data showing the changes of IgA and IgG concentration after AlHV-1 challenge were shown in detail in Figure 6.4 and 6.5 respectively.

Paired t-test comparison using GraphPad Prism v5, revealed that animals challenged with AlHV-1 had a significant effect on the IgA concentration in the vaccinated group 4 and 5 having significant differences with $p < 0.05$ with mean±(SD) of 3.18±0.7 g/L and 6.60±0.6 g/L respectively. However for IgG concentration, only group 5 shows a significant difference in post challenge having mean±(SD) of 3.30±0.5 g/L but not for group 4 ($p=0.09$) with mean±(SD) of 1.70 ±0.9 g/L.

In contrast, the mucosal antibody concentration in the disease animals (group 6) post-challenge show no significant difference in IgA concentration [$p=0.05$; mean±(SD)=3.13±0.4 g/L] but increased significantly for IgG concentration in NS post challenge [$p<0.05$; mean±(SD)=2.18 ±0.9 g/L].

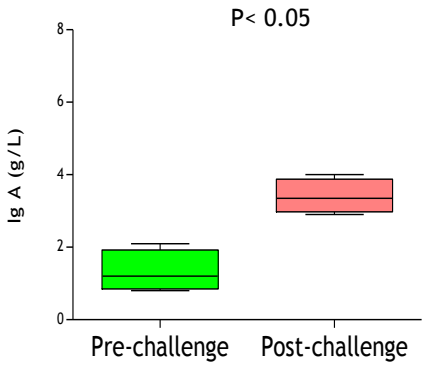
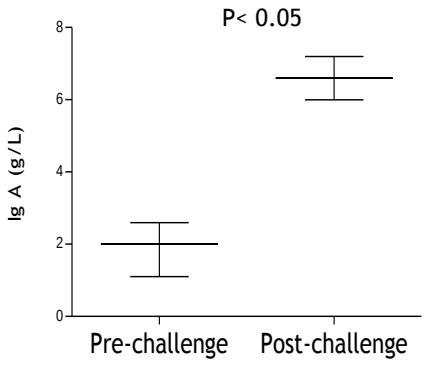
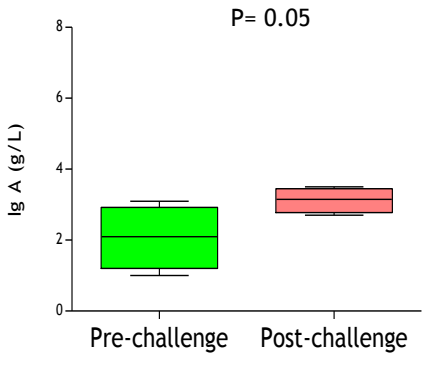
	Treatment type	Treatment route (primary/booster)	Challenge route	IgA (g/L)
Group 4 (n=4)	Att. virus	IM/IN	IN	
Group 5 (n=3)	Att. virus	IM/IM	IN	
Group 6 (n=4)	PBS	IM/IM	IN	

Figure 6.4: Concentration of NS IgA (g/L) response from cattle challenge intranasally with AlHV-1.
Data comparison of median NS IgA concentration pre and post-challenge by paired t-test. Att=attenuated; PBS=phosphate buffer solution; IM=intramuscular; IN=intranasal

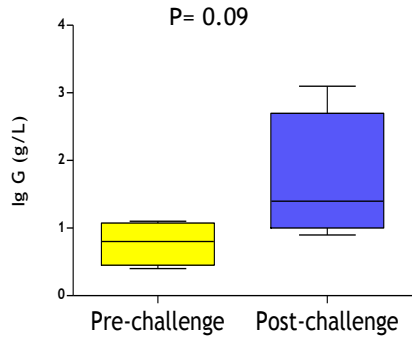
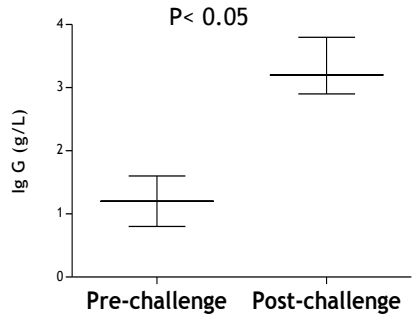
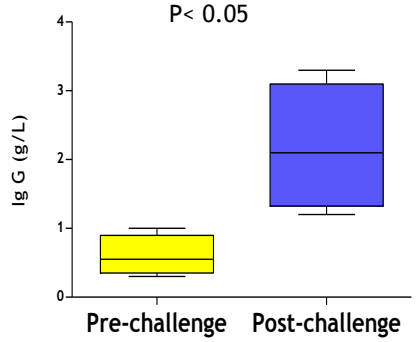
	Treatment type	Treatment route (primary/booster)	Challenge route	IgG (g/L)
Group 4 (n=4)	Att. virus	IM/IN	IN	
Group 5 (n=3)	Att. virus	IM/IM	IN	
Group 6 (n=4)	PBS	IM/IM	IN	

Figure 6.5: Concentration of NS IgG (g/L) response from cattle challenge intranasally with AlHV-1.
Data comparison of median NS IgG concentration pre and post-challenge by paired t-test. Att=attenuated; PBS=phosphate buffer solution; IM=intramuscular; IN=intranasal

6.3.4 DiGE analysis

All three CyDyes satisfactory bound to the nasal protein as evidenced by the 9 Cy images, of which 3 were the Cy2 bound internal standard. Figure 6.6 shows all 9 of the spot maps images created from the 3 DiGE gels as well as the 3 preparatory gel images representing the pooled healthy, disease and vaccinated group (Figure 6.7). Figures 6.8 details an example of DiGE gel image from healthy and disease group, with molecular weight and isoelectric point markers indicated together with labelled protein spots.

In DeCyder v.7.0, all DiGE images and Coomassie stained preparative gel images were analysed in the differential in-gel analysis (DIA) module. Total of 432 spots were detected in DIA module after the area of interest and the exclusion parameters were defined. Of the 432 spots that were identified, 71 were matched across the gels. One-way ANOVA was used in the BVA module to identify spots which were expressed significantly different between the 3 DiGE gels. A significance threshold of $P \leq 0.05$ was used and the false discovery rate was applied (Burchmore, pers. comm. 2014).

A total of 20 spots were differentially expressed in bovine NS in comparing the disease and vaccinated group as a result of AlHV-1 challenge. Eight spots showed increased and 12 spots decreased in expression in the disease group compared to the healthy group. Whereas for the vaccinated group, 11 spots showed increased and 9 spots decreased in expression in compared to the healthy group.

Table 6.4 lists the proteins that were detected in the spots that were differentially expressed in the disease and vaccinated group as a result of the AlHV-1 challenge. Using the log standardised abundance it was possible to plot the changes in spot volume in the disease and vaccinated group and compare their changes to results from the healthy group. Figure 6.6 – 6.8 depict the graphs of the major protein spots that were differentially expressed (change in abundance) in the disease and vaccinated group as a result of the AlHV-1 challenge in comparison with the healthy group.

From the spots that varied in expression within the disease and vaccinated group, 4 major spots were increased (spot 267: IgA; spot 380: IgG; spot 68: α 2-macroglobulin and spot 313: odorant binding protein) and another 4 major spots decreased (spot 271: apolipoprotein-A1; spot 240: fibrinogen beta chain; spot 347: hemopexin and spot 57: ceruloplasmin) in expression in both of the group and were selected to further illustrate the changes in expression post challenge with AlHV-1 are shown in Figure 6.12 and 6.13 respectively. In addition, Figure 6.14 shows the other 4 major spots expression that

were decreased in the disease group but increased in the vaccinated group (spot 320: haptoglobin; spot 244: complement C3; spot 352: serotransferrin and spot 451: cathelicidin).

The protein accession numbers were converted to gene ontology (GO) terms using NCBI and UniProt to identify each protein and subsequently their respective molecular and biological functions of the proteins that were affected as a result of AlHV-1 challenge. The GO terms for all the proteins were grouped and displayed in percentage values on a pie chart. Figure 6.15 and 6.16 represent all the proteins that were either increased in expression or decreased in expression for the disease and vaccinated group respectively.

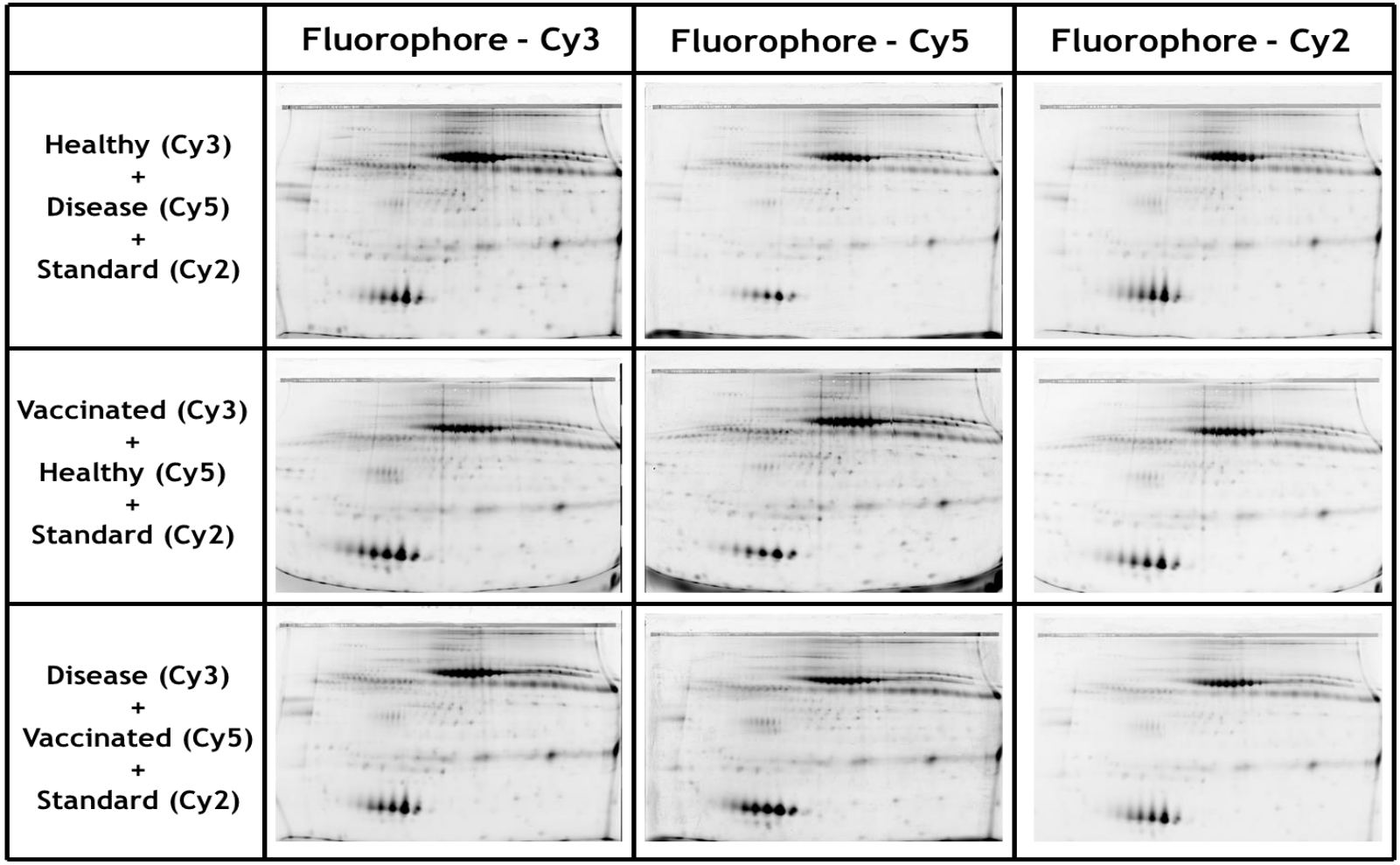


Figure 6.6: Nine spot map images from 3 DiGE gels consisting of pooled NS assigned according to Figure 6.1
Approximate peak wavelengths of excitation and emission values used to view Cyanine fluorophores (Excitation wavelength/ Emission wavelength) given in nanometres (nm). Fluorophore Cy3= (550/570); Fluorophore Cy5= (650/670); Fluorophore Cy2= (492/510).

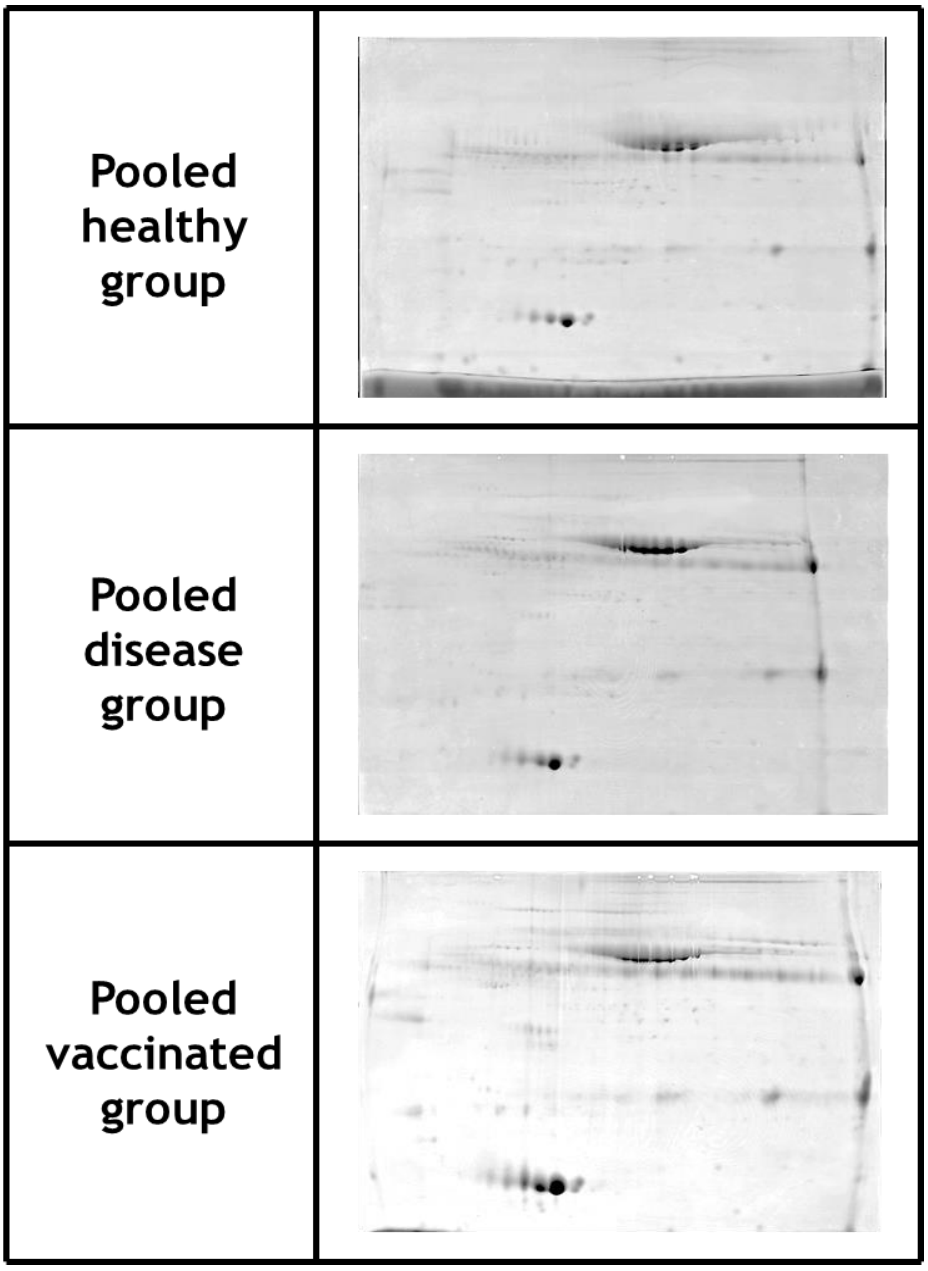


Figure 6.7: Three preparatory gel images stained with Coomassie blue representing the pooled healthy, disease and vaccinated group and were used to pick the spot that were differentially expressed for MS analysis and protein identification.

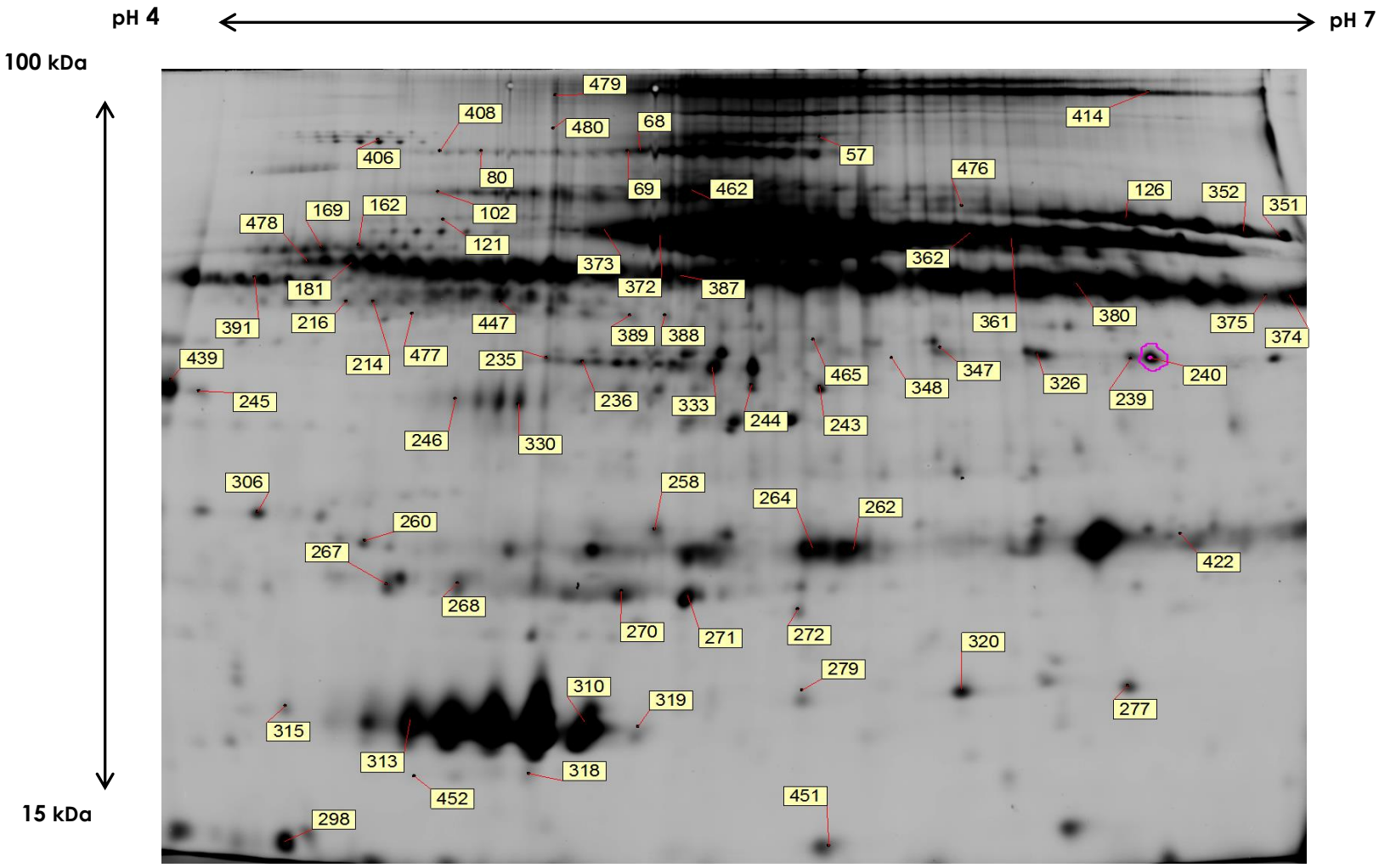


Figure 6.8: Spot map DiGE image of NS samples from the healthy and disease group.

Spot	Protein name	p value	MS Accession[a]	Theoretical Mw (kDa)	MOWSE score	Peptide matches	Protein function[b]	Biological process[b]
240	Fibrinogen beta chain	0.0045	CAA23444	57	722	112	APP, fibrin non-active form; involved in blood coagulation, cellular and matrix interactions, endothelial cell proliferation, the inflammatory response	Defence
372	Albumin	0.0049	AAA51411	71	1661	228	APP; main serum protein, transport of different molecules, responsible for the oncotic pressure, antioxidant properties	Transport
271	Apolipoprotein A1	0.0044	AAB21444	30	368	74	APP, main protein component of HDL; phospholipid, reverse cholesterol transport, LCAT cofactor, anti-inflammatory	Lipid metabolism
57	Ceruloplasmin	0.035	XP_592003	121	434	40	APP, major copper-binding protein; no clear role in copper transport, proposed functions: iron(II) oxidation, cellular iron levels	Transport
391	Kininogen	0.00055	AAI05500	70	449	85	Anticoagulant/anti-inflammatory properties, inhibition of vascular cell adhesion/ migration/ proliferation/ differentiation	Defence
347	Hemopexin	0.018	NP_001029784	52	342	63	APP, major heme-binding protein; toxic heme scavenger and transporter to the liver, heme iron recycler, antioxidant effect	Transport
126	Lactoferrin	0.0088	AAI16052	80	1107	149	APP, iron-binding glycoprotein; iron transport, antimicrobial/anti-inflammatory/immune system modulation	Transport
244	Complement C3 fragments	0.031	CAJ31249	68	554	110	Involved in complement cascade	Defence
320	Haptoglobin	0.0069	NP_001035560	46	383	60	APP; hemoglobin-binding protein, iron elimination control, antioxidant/anti-inflammatory role, immunomodulation	Transport

451	Cathelicidin	0.029	CAA70616	18	412	59	Antimicrobial properties	Defence
352	Serotransferrin	0.016	NP_803450	80	1404	147	APP, iron-binding protein; iron transport and distribution, antimicrobial activity, involved in growth, differentiation and cytoprotection	Transport
380	IgG2 heavy chain	0.036	AAB37380	51	749	58	Immunoglobulin fragment; antigen recognition	Defence
68	Alpha 2-macroglobulin	0.0058	NP_001103265	167	621	85	APP; proteases inhibition and clearance, inflammatory regulation	Defence
313	Odorant binding protein	0.0022	P07435	18	579	97	Binds a wide variety of chemical odorants, sensory transduction, olfaction	Transport
267	IgA light chain	0.016	XM_005219676	25	343	45	Immunoglobulin fragment; antigen recognition	Defence
478	Serpin	0.0046	Q1JPB0	46	578	78	Cysteine or serine protease inhibitor family; neutrophil proteases Regulation	Defence
465	Plasminogen	0.033	NP_776376	91	317	104	APP, plasmin non-active form; serine protease, anticoagulant properties by fibrinolysis, cell migration enhancer, inflammatory response regulation	Defence
264	Igλ light chain	0.0085	AAK84156	25	716	45	Immunoglobulin fragment; antigen recognition	Defence
80	Complement C3	0.0004	AAI12453	189	530	83	APP; involved in complement cascade	Defence
422	IgG1 heavy chain	0.016	AAB37381	36	697	60	Immunoglobulin fragment; antigen recognition	Defence

Table 6.4: Proteins differentially expressed in NS between healthy, disease and vaccinated group.
p values <0.05 was considered significant different in protein expression, and a list of proteins identified by MS, together with theoretical molecular weights, peptide matches, accession number, MOWSE score, protein function and biological processes. APP = Acute phase protein. [a] Database accession numbers: SWISS-PROT (http://us.expasy.org/), ncbi (http://www.ncbi.nlm.nih.gov/). [b] Protein Function and Biological Process, if not stated otherwise, according to SWISS-PROT (http://us.expasy.org/uniprot/).

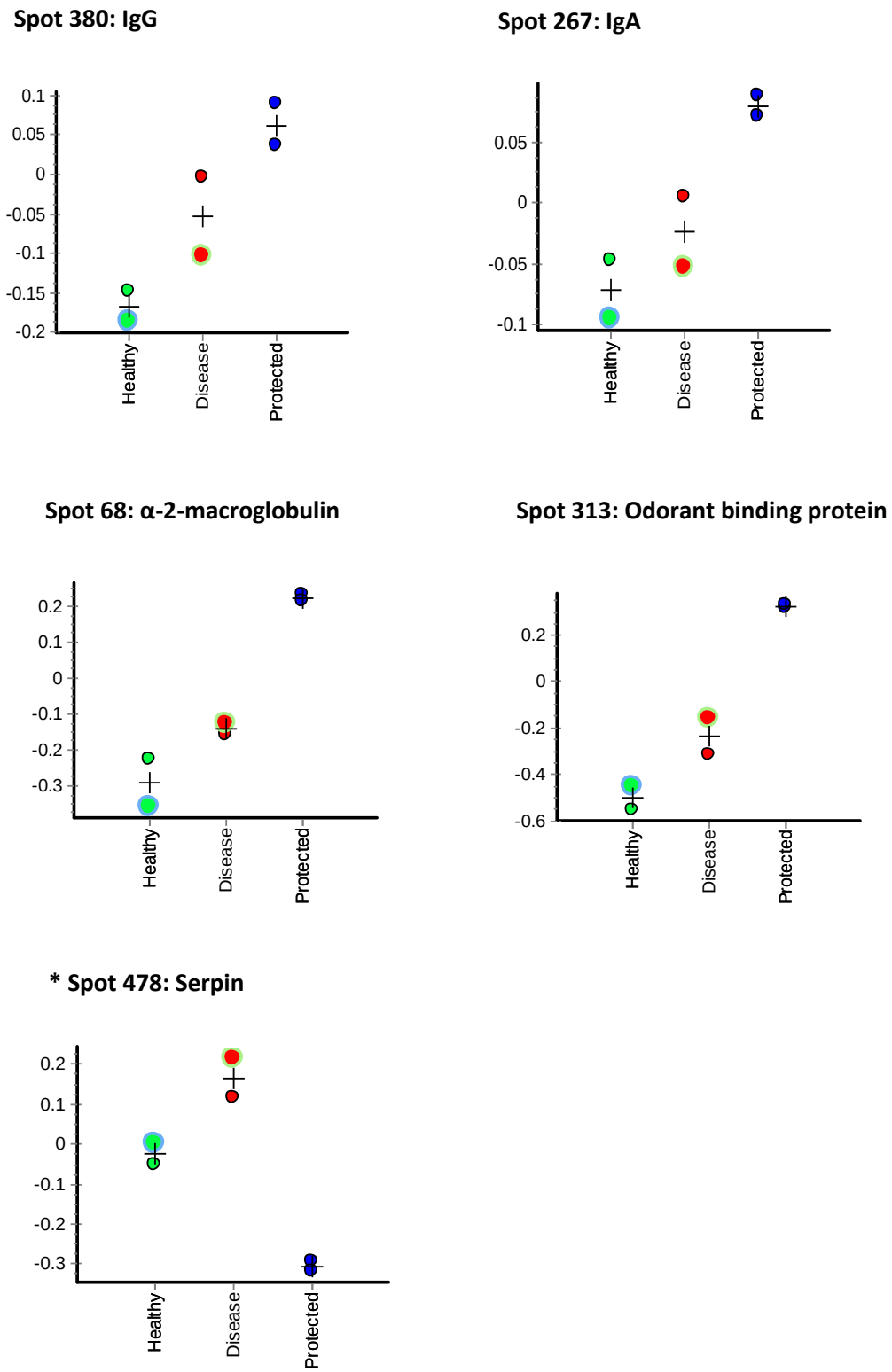


Figure 6.9: Spots that increased in abundance in disease and vaccinated (protected) group.
*Spot 478: Serpin was decreased in abundance in vaccinated cattle

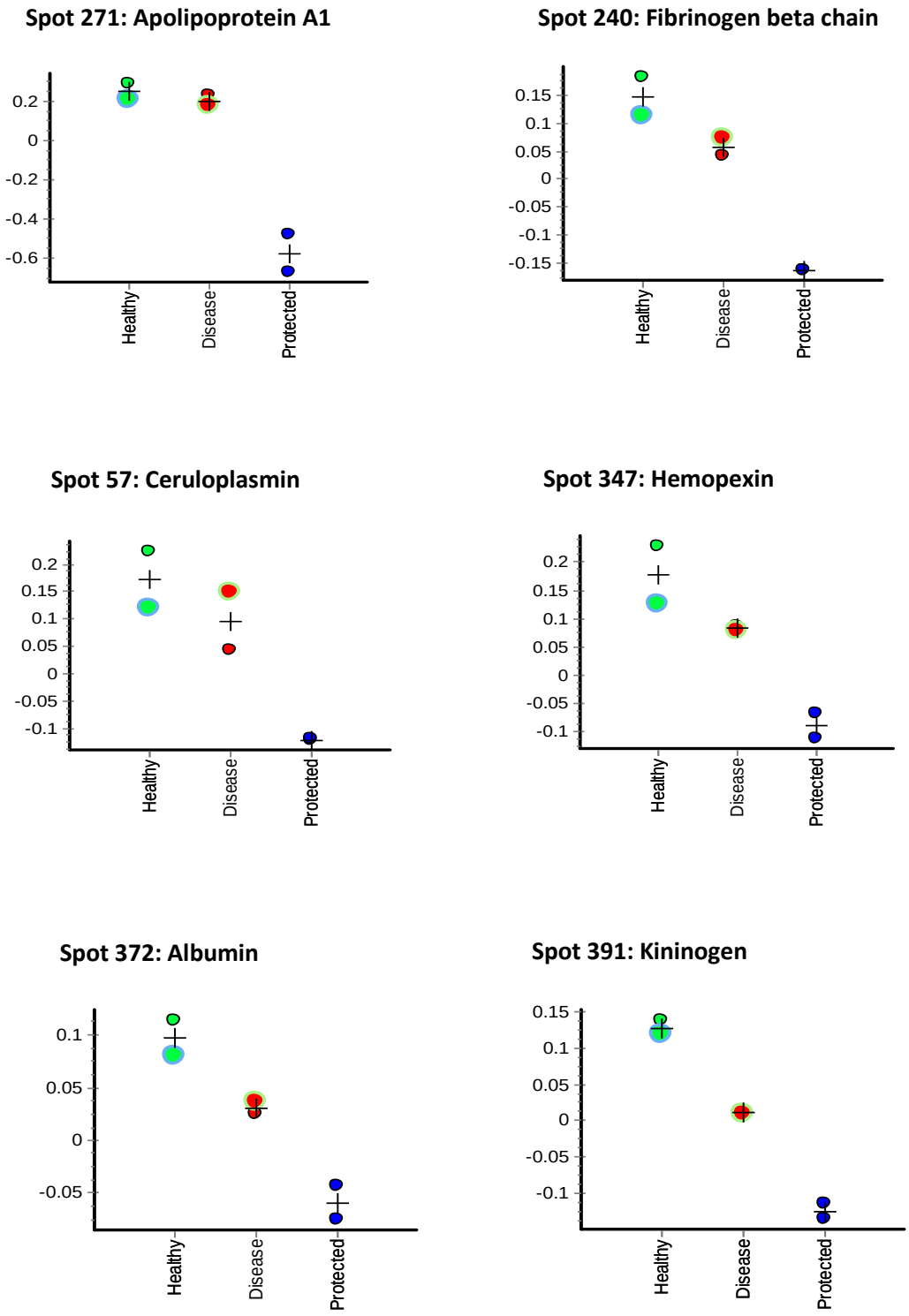


Figure 6.10: Spots that decreased in abundance in disease and vaccinated (protected) group.

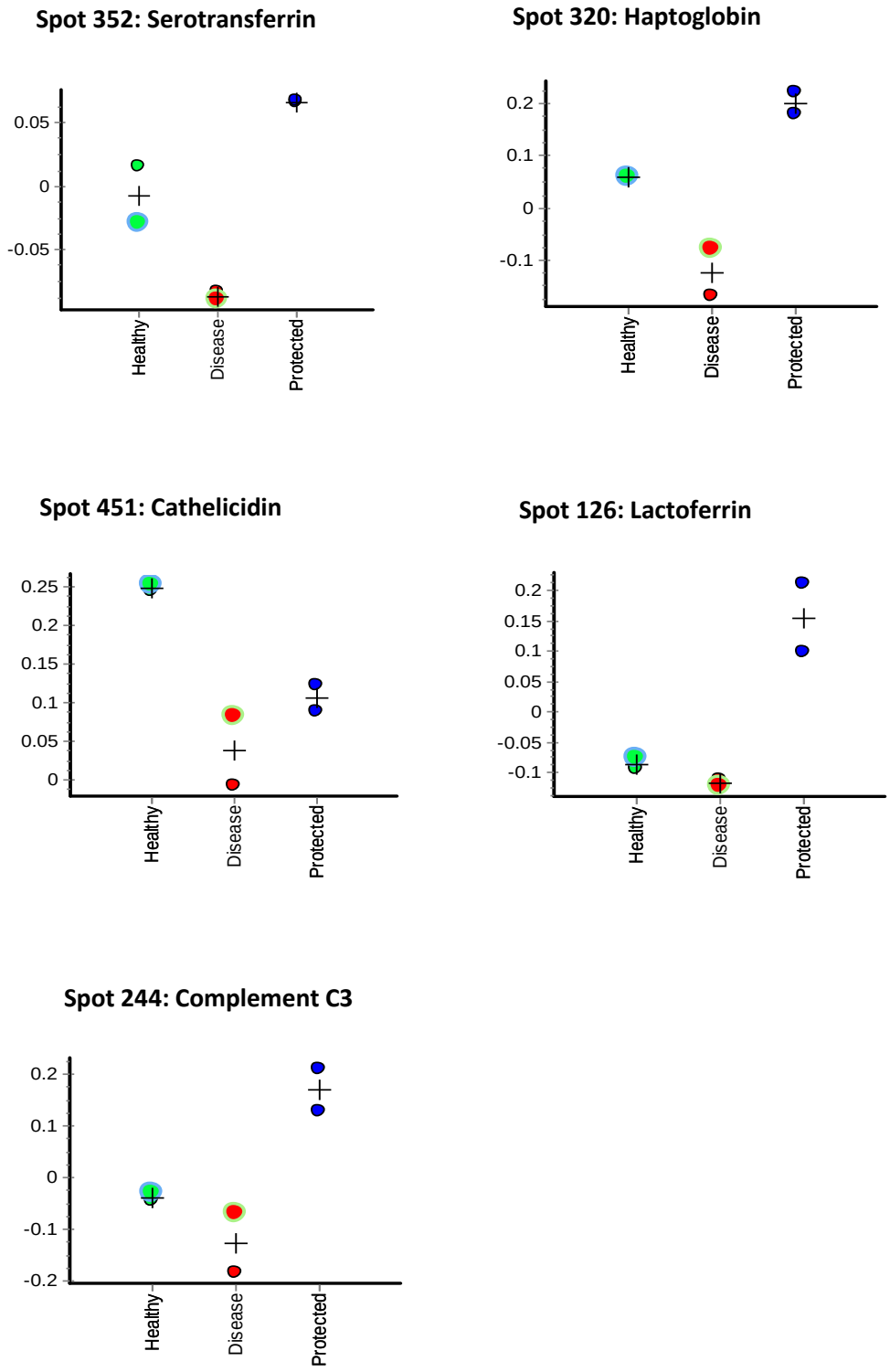


Figure 6.11: Spots that varied responses but had significant change in comparison to healthy animals.
Apart from spot 451: Cathelicidin that decreased in abundance in disease and vaccinated (protected) group, other spots show decrease in abundance in disease group but increased in abundance in vaccinated (protected) group.

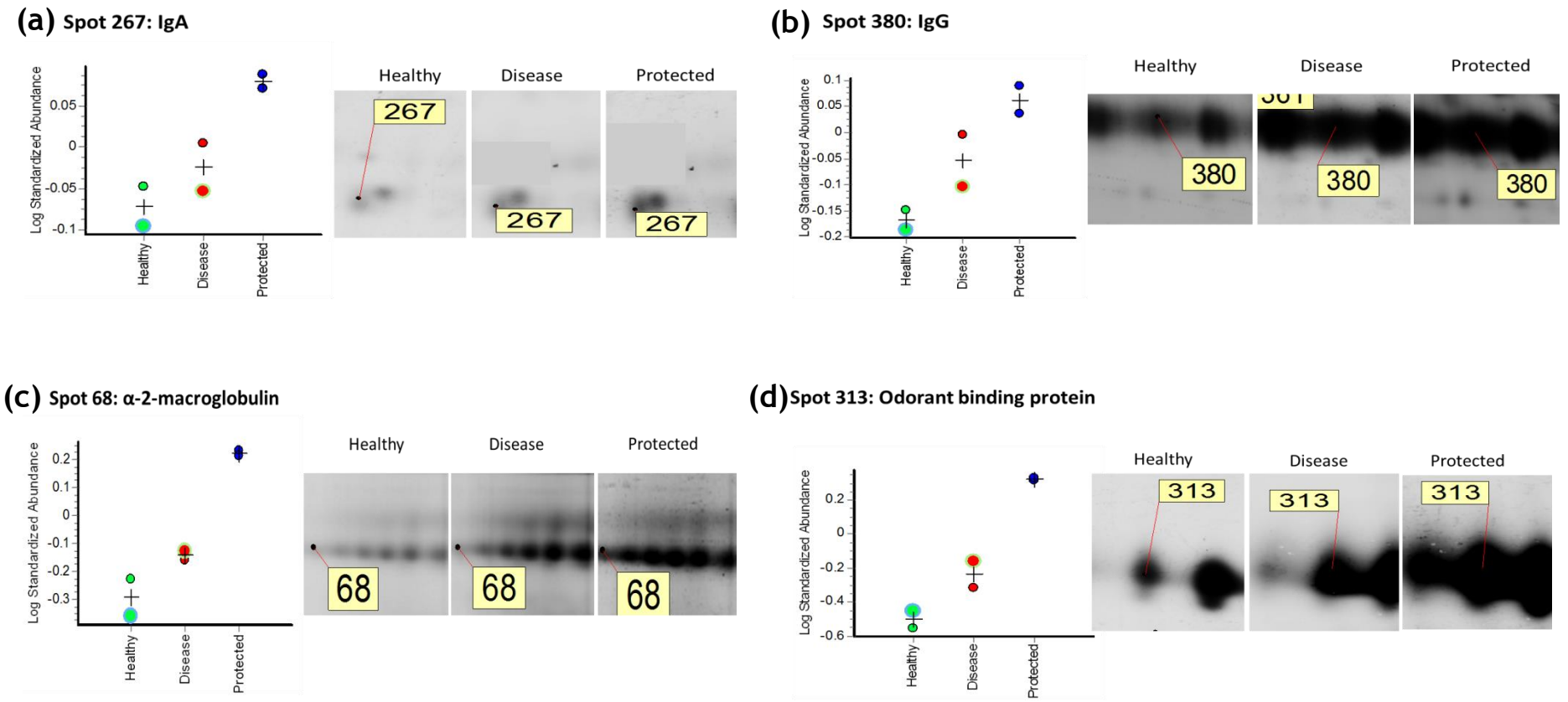


Figure 6.12: Selected spots are highlighted alongside graphical presentation showing increased in log standardised abundance in the disease and vaccinated (protected) in compare to the healthy group.
(a) IgA, (b) IgG, (c) Alpha 2-macroglobulin and (d) Odorant binding protein.

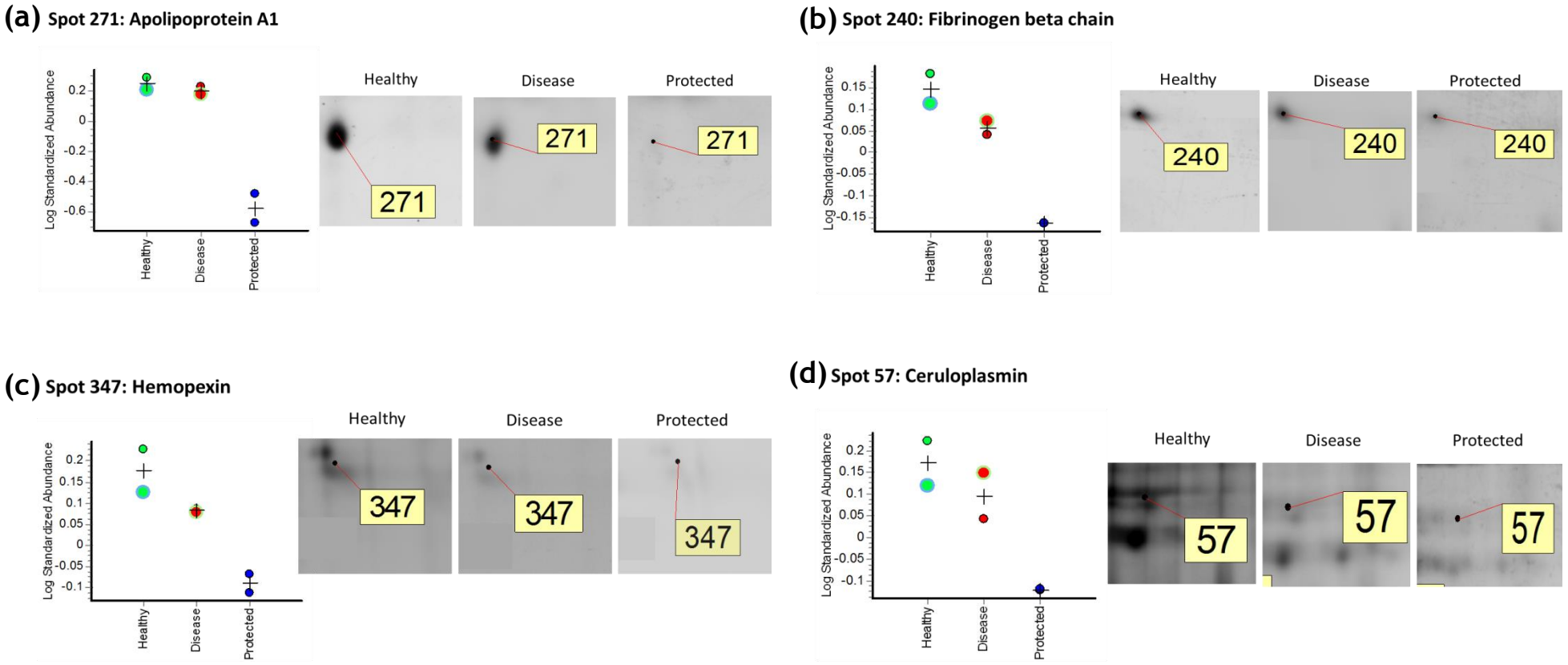


Figure 6.13: Selected spots are highlighted alongside graphical presentation showing decreased in log standardised abundance in the disease and vaccinated (protected) in compare to the healthy group.
(a) Apolipoprotein A1, (b) Fibrinogen beta chain, (c) Hemopexin and (d) Ceruloplasmin

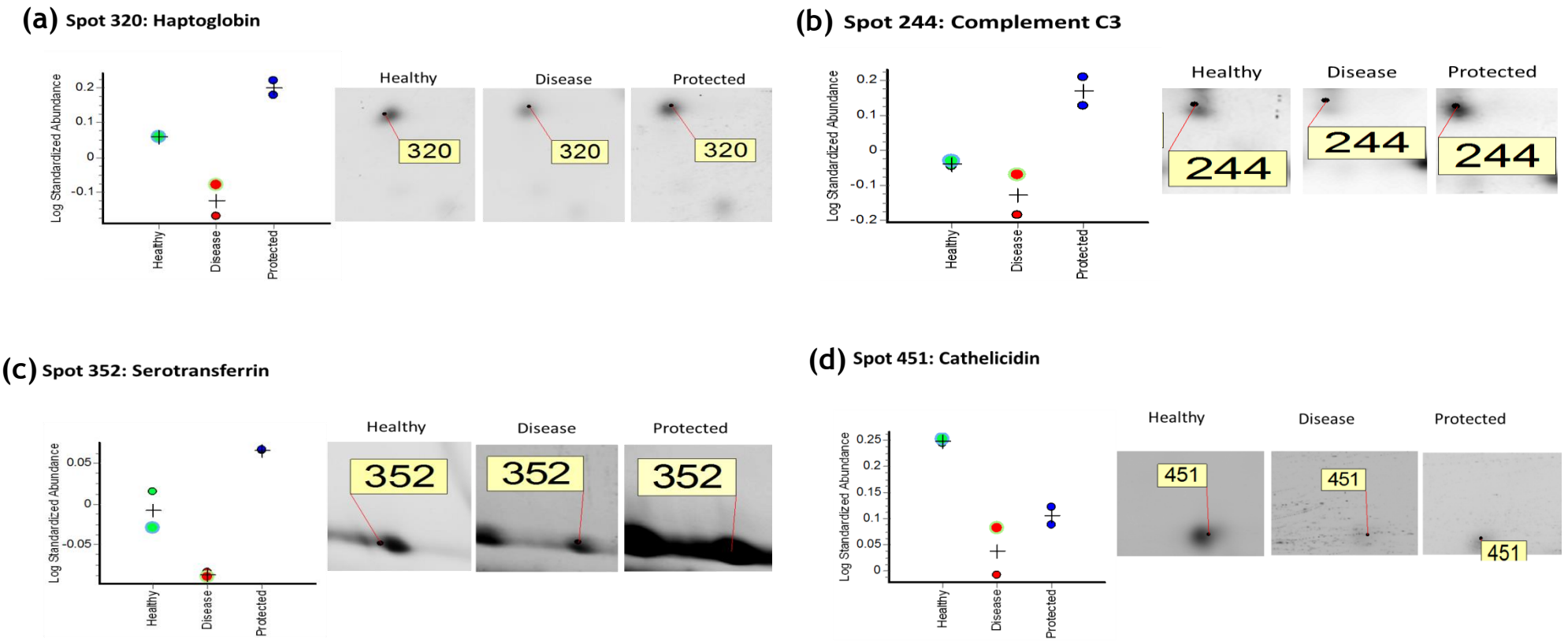


Figure 6.14: Selected spots are highlighted alongside graphical presentation showing decreased and increased in log standardised abundance in the disease and vaccinated (protected) respectively in compare to the healthy group.
(a) Haptoglobin, (b) Complement C3, (c) Serotransferrin and (d) Cathelicidin

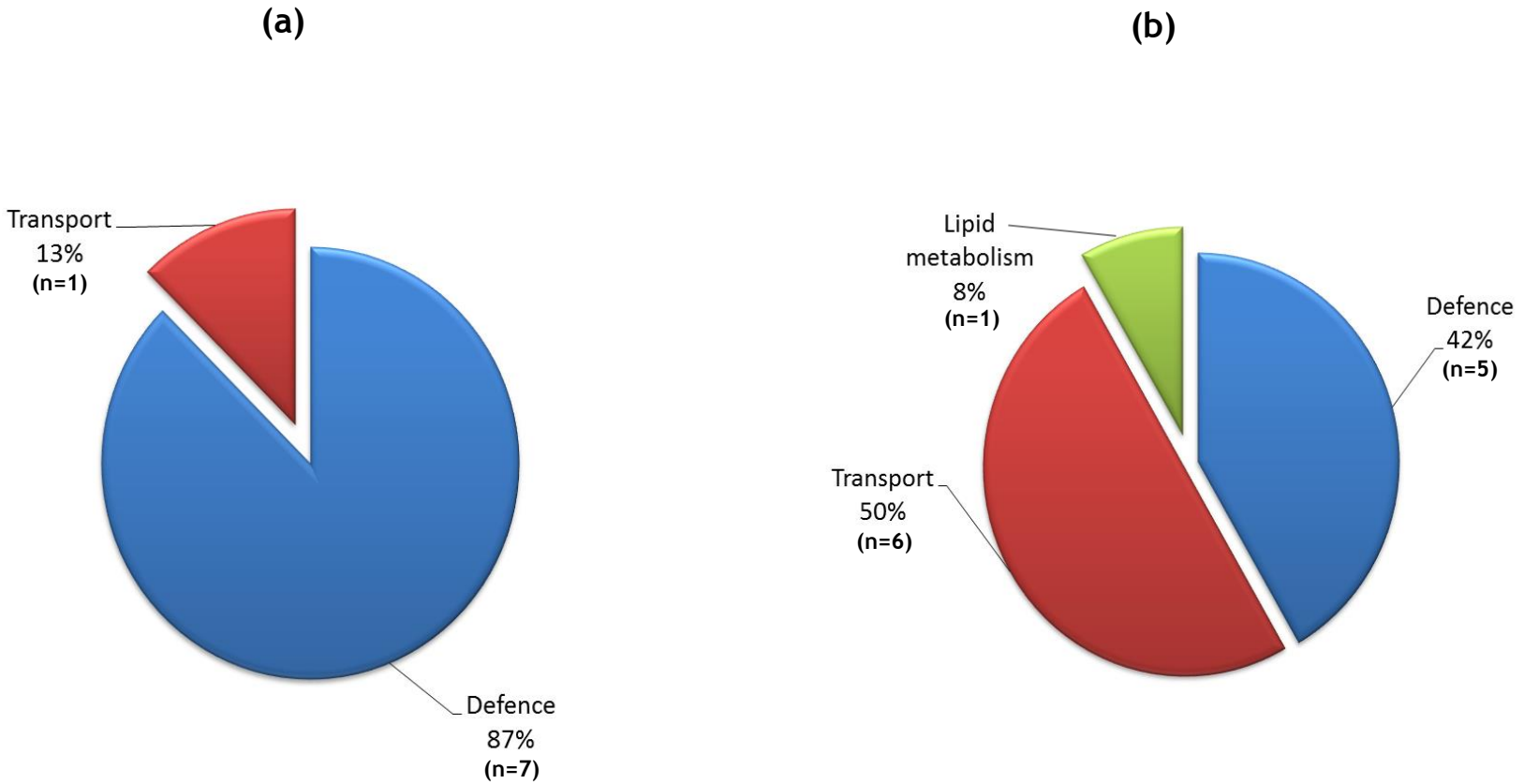


Figure 6.15: Using gene ontology annotations, the biological functions of all of the proteins (n=20) from the spots that increased (a) and decreased (b) in disease group were compared.
Proteins biological function according to SWISS-PROT (http://us.expasy.org/uniprot/).

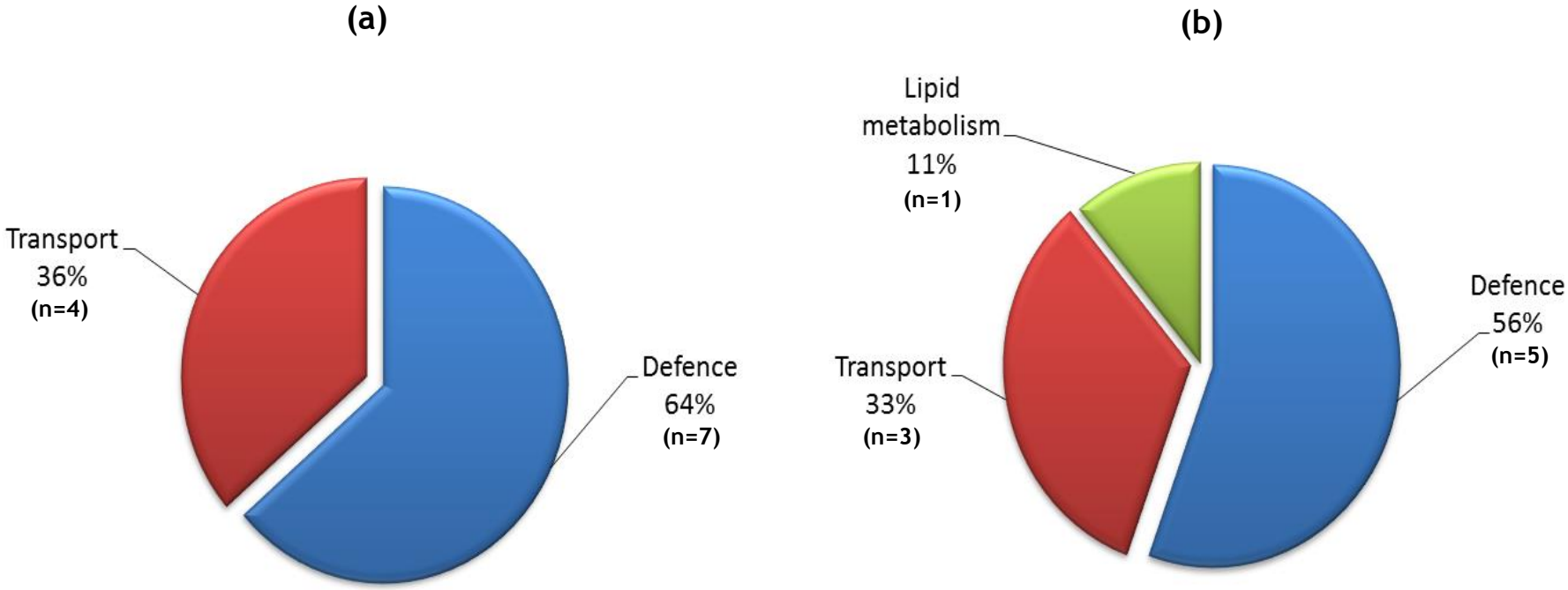


Figure 6.16: Using gene ontology annotations, the biological functions of all of the proteins (n=20) from the spots that increased (a) and decreased (b) in vaccinated group were compared.
Proteins biological function according to SWISS-PROT (http://us.expasy.org/uniprot/).

6.4 Discussion

Effect of NSAP activity, IgA and IgG concentration in response to AlHV-1 intranasal challenge

The NS sample used in this chapter derived from a previous immunization study showing that a vaccine based on attenuated AlHV-1 protected cattle challenged intranasally with pathogenic AlHV-1 C500 from developing clinical MCF (Haig et al., 2008). The intranasal challenge of AlHV-1 given to the animals that were or were not given protection had no effect on the AP activity present in the NS. No significant difference between the pre-challenge and post-challenge NSAP activity was observed within the 3 tested groups (group 4, 5 and 6) and there was no significant association between immunised cattle and NSAP activity. However the post challenge sample was taken 14 days after challenge to fit with the objectives of the original experiment and it is possible that changes in AP occurred in the time immediately post challenge. There may also have been degradation of AP during storage (around 5 years) even though kept at -80oC. It would be valuable to repeat the investigation with fresh samples taken more frequently post-challaenge.

There was a significant increase of nasal mucosal antibodies in the NS of animals from group 5 indicating that cattle given both prime vaccination and booster by IM increase the IgA and IgG concentration in NS when challenged with AlHV-1 intranasally. In contrast, only the IgA concentrations were significantly increased in the NS from group 4 animals whereas no significant change in the IgA and IgG concentration were observed in the NS collected from the animals in group 6.

The increase in IgA and IgG antibodies expression in NS resulting from intranasal challenge of AlHV-1 could play a role in protection against MCF by contributing additional antibodies present in the nasal mucosa thereby neutralizing virus at the portal of entry. Furthermore, Peri et al. (1982) and Waldman et al. (1973) have reported that respiratory viruses given challenge by oral route in rabbits, mice or man have also led to the increase of IgA response in NS. Studies in sheep have shown that the numbers of IgA antibody containing cells in the upper respiratory tract could be enhanced following intratracheal boosting of intraperitoneally primed animals (Scicchitano et al., 1984). Immunization studies by Burrough et al. in 1982 showed that intranasal IBR-PI3 virus vaccines were unable to stimulate any significant cell-mediated or humoral antibodies in the serum. However a later work by Saoud et al. (2004) reported that trivalent vaccine for IBR, PI3 and *P. multocida* type B given intranasal had

stimulated the development of satisfactory level of circulating and mucosal antibodies in the NS.

Although the intranasal route may provide the alternative route of MCF immunization, this study has shown that the group of animal that received primary vaccination and booster of attenuated AlHV-1 vaccine both given via IM route has led to a better outcome with 100% survival rate in comparison to the group that receive booster given by the intranasal route (Table 6.3). The result also showed that immunization given by the former regime produced a higher IgA and IgG concentration in the NS in compare to the latter regime (Figure 6.4 and 6.5).

In an earlier report by Howard et al. (1986), replication of *M. bovis* in the bovine respiratory tract elicited IgG and IgA responses in tracheobronchial washings, similar to the responses present in NS seen in the present study. These similarities may relate to the invasiveness of *M. bovis* which causes extensive lung lesions which was also typically seen in MCF infections (Reynolds et al., 1985; Howard et al., 1986; Haig et al., 2008 and Russell et al., 2009). Invasive AlHV-1 given at a fatal dose as demonstrated in the immunization study in this chapter might evoke IgG responses since IgG plasma cells predominate in the lower respiratory tract (compared with IgA cells in the upper tract, Morgan et al., 1981). Alternatively IgG antibodies may be partially serum-derived in response to the inflammation and damage produced by invasive infections of AlHV-1 given challenge intranasally.

Protein identification and changes in protein expression

Even though the healthy, disease and vaccinated NS show a very similar protein composition, as seen from their gel images and their ontology analysis (Figure 6.6 and Table 6.4), some marked differences in expression levels and patterns were observed (Figure 6.9 - 6.14) by DiGE analysis. Of the proteins that were found to decrease in the unvaccinated group of animals when challenge with AlHV-1, it was clear from the gene ontologies obtained from UniProt that most of the proteins associated with host defence mechanism were increased in expression. Mucosal antibodies like IgA, IgG, alpha 2-macroglobulin and other proteins involved in the host innate defence mechanism constitute 87% of the total proteins that were up regulated in the NS from the disease group compared to 56% of up regulated proteins seen in the vaccinated group. The rest of the NS proteins were mainly involved in transporting biomolecules such as odorant binding protein involved in nasal sensory transduction and binding to a wide variety of

chemical odorants. Other proteins found to decrease in expression were mainly serum APPs such as apolipoprotein A1, albumin, ceruloplasmin, hemopexin, fibrinogen as well as proteins having antimicrobial and anti-inflammatory properties like cathelicidin, serotransferrin and lactoferrin.

Most of the proteins that involve in local defence mechanism such as immunoglobulins and antimicrobial proteins like serotransferrin and lactoferrin were up regulated in the vaccinated group hence suggesting that immunizations given via IM were able to stimulate the increase of local innate protection in the nasal mucosa. However the majority of APPs such as apolipoprotein A1 were decreased in expression in comparison to both healthy and disease group. Although cathelicidin is an antimicrobial protein in NS showing a higher protein expression in the vaccinated group than in disease, its expression was below that seen in the healthy group.

Alpha 2-macroglobulin functions as a broad-spectrum inhibitor of serum proteases and those produced by pathogens. Although it is a strongly positive APP in rats (Jinbo et al., 2002 and Strnad et al., 2000), in mice both increased (Isaac et al., 1999) and decreased (Kristiansson et al., 2007) levels of the protein have been found associated with different inflammatory processes, as well as in humans (Makis et al., 2000 and Chowdroska et al., 2004). In this study, alpha 2-macroglobuline (spot 68, Figure 6.7) was slightly increased in NS from the disease group and almost 4-fold increase in abundance in the vaccinated group. Antibodies constitute essential components of the mucosal immune system of vertebrates and also the main humoral immunity commonly present in the serum. In case of infection immunoglobulin levels in the organism are increased in order to fight the pathological agent. This is clearly shownin the DiGE investigation by the increased of IgA and IgG abundance in the NS post-challenge where the vaccinated group having a higher increased compare to the disease group. This findings correlate with the IgA and IgG results obtained by ELISA as shown in section 6.3.3.

The most abundant protein in NS which is albumin has multiple functions. It transports different molecules, such as metals, fatty acids or drugs, and is the major protein responsible for the biomolecule transportation. Also, in general it represents the predominant antioxidant in serum (Kouoh et al., 1999 and Roche et al., 2008). Furthermore, albumin is a negative acute phase reactant (Schreiber et al., 1982 and Gruys et al., 2005). This corresponds to the change observed at spot 372 (Figure 6.10) which represents the main albumin spot, the albumin level is decreased in NS from the

disease group and even lower in abundance seen in the NS from the vaccinated group. Visual comparison of the major protein spot (albumin) on the DiGE gels between the healthy, disease and vaccinated group also confirmed this finding (Figure 6.6).

Another serum transporter that can multi-task is transferrin. Although its primary role is to transport iron, in addition it has been reported that it possesses antimicrobial properties and is implicated in growth and differentiation activities. Like albumin, serotransferrin (spot 352 in Figure 6.8) is considered as a negative APP in cattle. DiGE results showed decreased in abundance in NS from the disease group however serotransferrin was significantly increased in the vaccinated group. This probably due to the effect of immunization receive prior to AlHV-1 challenge which resulted to increase of serotransferrin in NS although further study need to be done to conform this theory.

Hemopexin (spot 347 in figure 6.8) binds and scavenges heme released by the turnover of heme proteins, thus preventing heme mediated oxidative stress and heme-bound iron loss. Although its synthesis is induced under inflammatory conditions, the protein was decreased in the disease and vaccinated group probably due to the increased binding activity due to significant degradation of heme containing compounds in NS. The main protein component of HDL, apolipoprotein AI (spot 240 in Figure 6.8), is involved in phospholipids and reverse cholesterol transport. It also functions as a cofactor for lecithin-cholesterol acyltransferase (LCAT) and possesses anti-inflammatory and anti-coagulant properties (Chen and Albers, 1985 and Andersson, 1997). Additionally, this protein has been suggested to inhibit toxicity (Ma et al., 2004). Apolipoprotein A1 usually categorized as a negative APP and the NS expression in this study was also significantly reduced both from the disease and vaccinated group post-challenge with AlHV-1.

Apart from studies of lactoferrin showing its major function in milk, this protein is also a characteristic mucosal protein with antimicrobial and anti-inflammatory properties. Lactoferrin present in the bovine NS is believed to be produced and secreted locally by the nasal exocrine glands whose biological function has been discussed in detail in Chapter 5. It has been often reported to rise in disease cases such as in mastitis (Hagiwara et al., 2003 and Eckersall et al., 2006) and respiratory toxicity (Ghio et al., 1999). However in this study, lactoferrin expression was decreased in the disease group but increased almost 5-fold in protein abundance within the vaccinated group. As in serotransferrin, the increase in expression seen in vaccinated group might be related to the successful immunization effect against AlHV-1 given by the IM route. This however

needs to be further investigated to confirm exactly the specific pathway whereby the vaccine could stimulate the increase of lactoferrin expression in the NS.

In conclusion, this study reveals that as a result of intranasal challenge of AlHV-1, the bovine NS proteome from both disease and vaccinated group showed differential expression of a number of proteins. The comparable results also indicate that many of the responses to infection and inflammation in the NS are conserved within the nasal mucosa. The changes of NS protein expression as a result of MCF challenge and vaccination have not been reflected in other studies and together with the result from the previous chapter, this study shows that the majority of the NS protein present consist mainly of protein that are involved in innate immunity protection and transportation, whereby these NS proteins were differentially expressed in the disease and vaccinated cattle in response to AlHV-1 challenge.

Up to the present time, there are only a few proteomic studies of human NS and no published work was found to use proteomic technology as a tool to study the NS from animals. The used of DiGE has shown in this chapter to be able to successfully identify and measure the changes of NS protein expression in MCF challenged and vaccinated cattle. This would allow a more comprehensive understanding on disease progression and host response towards infection as well as the detection of potential biomarkers. However, the use of proteomic methods to investigate a novel biological fluid like NS requires much more work to be done. Proteomic methods such as DiGE could be used to analyse and examine the NS collected daily post infection to study progression on disease recovery. Furthermore, more work should be done to find a way to normalise the results data collected and also to observe if volume of NS production could affects the concentration or abundance of nasal proteins. In addition, most of the NS proteins detected and analysed in this chapter consist of soluble proteins with Mw <200 kDa hence preventing the detection of larger proteins such as mucins that do not get into the electrophoresis gels. The use of larger pore acrylamide gels to separate bigger and multimeric protein complexes may allow for the detection of these proteins.

The DiGE system is a variant of 2-DE which offer the possibility of including an internal standard making comparison of gels easier and quantification of proteins more accurate (Marouga et al., 2005). The DiGE technique, first described by John Minden's lab (Unlu et al. 1997), circumvents many of the issues associated with traditional 2-DE such as reproducibility and analytical problems associated with gel to gel variation and a limited dynamic range; all of which severely hamper a quantitative differential display

study (Lilley and Friedman, 2004; Friedman and Lilley, 2008). Utilising DiGE technology allows for a more accurate and sensitive quantitative proteomic study because DiGE as shown in this study has proven to be a powerful technology for differential display proteomics where the individual abundance changes for thousands of intact proteins can be simultaneously monitored in replicate samples over multiple variables with statistical confidence (Lilley and Friedman, 2004; Friedman and Lilley, 2008).

However, some common drawbacks of 2-DE would still occur including limited sample capacity, insufficient detection of low-abundant and hydrophobic membrane proteins as well as low visualization sensitivity (Monteoliva and Albar, 2004). Alternatively the non-gel based technique liquid chromatography–mass spectrometry (LC/MS) has the potential to identify highly complex samples for large scale proteome analysis such as the human plasma proteome (Fujii et al., 2004). The work of Martin and Synge (1941) had suggested that small sorbent particles and pressure could produce fast LC techniques. Since then, LC/MS has seen enormous growth in clinical laboratories especially during the last 15–20 years. It offers analytical specificity superior to that of immunoassays or conventional high performance/pressure liquid chromatography (HPLC) for low molecular weight analytes and has higher throughput than gas chromatography-mass spectrometry (GC-MS; Grebe and Singh, 2011).

Therefore, LC/MS could offer a great alternative to overcome many 2-DE limitations. However, LC/MS and similar “shotgun” methodologies are non-quantitative based proteomic hence for quantification of potential biomarkers in bovine NS using this technology, quantitative methods based on protein labelling (such as iTRAQ or SILAC) or on unlabelled approaches should be considered for future studies.

Chapter 7

General Discussion

Chapter 7

The aims of this thesis were outlined in Chapter 1 and the relevant conclusions have been detailed in the appropriate chapters. This chapter serves to summarise the most significant findings from preceding chapters and to highlight the areas which have been recognised as worthy of future research.

Bovine respiratory disease (BRD) has the greatest economic impact towards the cattle farming industry and up to the present, pneumonia is a leading cause of loss to the cattle industry in the United States and Europe (Griffin, 1997 and Ackermann et al., 2010). An estimated 1.9 million animals (Nicholas and Ayling, 2003 and Nicholas, 2011) are affected by BRD each year in the UK with costs estimated at around £60 million annually (NADIS, 2007). Many efforts have been made to identify the primary respiratory pathogen responsible based upon gross necropsy and histopathological lesions (Tegtmeier et al., 1999 and Gagea et al., 2006). However, given a typical interval of several days to weeks between disease onset and death (Belknap, 1993) it is uncertain how post mortem findings and isolation of etiologic agents solely could be beneficial for early detection and prevention of BRD. Therefore, there is a need to understand exactly how the host innate immunity responds towards the onset of infection and is involved in protecting the animal.

Aerial transmission through contaminated NS is an important route of infection for BRD (Taylor et al., 2010 and Fulton and Confer, 2012) and the most common clinical sign observed in respiratory cases is excessive nasal discharges (Briggs and Frank, 1992 and Love et al., 2014). Although the etiopathogenesis of BRD is multifactorial and complex, all of the pathogens have the common route of infection which is intranasal. The nasal mucosa is the primary portal of entry for not only inspired air but also respiratory pathogens thus NS produced by the nasal mucosa can play a major role to protect the lower respiratory tract from inhaled microbes. The increase in production of NS during disease is known to be one of the protective innate responses against aerial borne infections.

Therefore, NS serves as a valuable source of biological sample to investigate how it acts as a protective barrier against respiratory disease. However, the application of using a NS sample for diagnosing or monitoring respiratory diseases in veterinary species is not common. Unlike human (Rubin, 2002; Ganz, 2003 and Laube et al., 2006), the clinical biochemistry of NS from animal has not been well characterised but could provide useful indicators of immune function and status. Therefore to use a biological fluid such as NS for diagnosing and monitoring disease or for the detection of biomarkers, it was necessary first to establish a reference range of analytes that are present in the NS of

healthy cattle. Prior to the investigation, there is no information on the composition of the bovine NS, possibly due to the perceived difficulty of collecting a sufficient volume of NS from healthy animals for analysis.

It was with this in mind that a practical and non-invasive method for the collection of bovine NS in substantial volume was developed which was described in detail in Chapter 2. Previous studies by Haig et al. (2008) described the collection of bovine NS on tampons and the secretion was removed by mechanical extraction with 60 ml syringe. However this method although allowed the collection of undiluted NS samples, the actual volume of NS removed from the tampon was low being between 1-3 ml, when compared to the actual NS absorbed by the tampon which could be assessed by means of weighing the tampon weight post collection.

This study has established a method of extracting NS from the absorbent of the tampon by centrifugation using modified collecting tubes (Figure 2.2), which have allowed up to 12 ml of NS to be collected at a time from each animal. This method allowed NS to be collected in substantial volume hence provide sufficient amount of biological samples for multiple analysis. Moreover, the requirement to apply non-invasive techniques to collect samples for experiments have been one of the primary aims in any study involving animal especially in veterinary research. The method described in this study is simple to perform, does not require any chemical restrain, is non-traumatic and causes only minimal to none discomfort to the animal. In contrast to other methods used for the collection of NS, such as nasal lavage or nasal swabs, which commonly involve diluting the fluid in buffer , this method has the advantage that NS collected is undiluted and thus contains high concentrations of biomolecules.

The biochemical analysis of 38 NS samples collected from healthy cattle described in Chapter 2 was carried out using an Olympus A640 analyser operated by Veterinary Diagnostic Services, University of Glasgow and the results were compare to standard bovine serum reference. Concentrations of total protein, albumin, calcium, phosphate, sodium and chloride were lower than the serum reference range while urea, creatinine, bilirubin and aspartate transaminase levels were comparable to serum reference ranges. Interestingly, 2 notable findings were seen in bovine NS. The mean (±SD) AP activity in NS was 1259±520 IU/L and was up to 15.7-fold higher at 80:1259 than the serum reference range for AP while the mean GGT activity was 67±21 IU/L and was up to 2.5-fold higher at 27:67 than the bovine serum GGT reference range. The components of NS had generally been associated with plasma exudation from the local blood vessel of nasal mucosa as the major source of constituent of its biomolecules. The extensive

differences in AP and GGT activity present in NS in compare to serum reference highly suggest that these enzymes were locally produced. In addition, since the NS was collected from healthy population, those findings reflect the natural composition of bovine NS and not a response towards any disease. Additionally, a preliminary study to compare the NSAP activity from cattle challenged with intranasal MCF virus (n=4) had showed that the mean NSAP activity from pre challenge animal was further increased by 3-fold in the NS collected post challenged.

There are no previous reports of the activities of these enzymes in NS being greater than that found in serum or plasma hence this novel discovery justified a further thorough investigation as described in this study. Whether the high activity seen was associated with the increase of NS production during disease or AP was being actively produced and secreted in response to local nasal stimulation are questions that still need to be addressed. The theory suggesting NSAP was a result of leakage from serum is not supported by the finding of a higher activity of AP in NS than in serum unless a mechanism to export the AP against a concentration gradient is present in this tissue. Subsequently, the molecular nature of nasal AP and whether this enzyme has the same characteristic and physiological function with the well-known bovine IAP would be addressed in Chapter 3 and 4.

This chapter have established a reference range of analytes that are present in the NS of healthy cattle. This data should lay the groundwork for future investigation on BRD such as studies on biomarker discovery involving bovine NS as the source of biological samples. The next logical step in this thesis was to examine the source of high AP activity reported in bovine NS.

Chapter 3 specifically dealt with the investigation of AP that was found to be present in bovine NS. The objectives were to 1) Determination of the tissue responsible for producing and secreting NSAP by histochemistry, 2) Comparison of nasal AP activity with other relevant bovine tissue, 3) Detection and quantification of AP concentration in NS and nasal mucosa and 4) Characterization of nasal AP isoenzyme with electrophoresis and isoelectric focusing (IEF).

Histochemistry using an AP specific stain showed strong AP activity was present in the luminal and basal ends of nasal epithelium and serous glands. Control experiments also confirm the findings where AP activity staining was abolished in the presence of levamisole which is a known non-specific AP inhibitor. Therefore, this finding confirmed

that the respiratory cell and the serous glands of the nasal mucosa are responsible for producing and secreting AP hence the presence of strong AP activity seen in NS. Apart from the findings shown in 3.3.3, there have been few studies involved in localising AP in the nasal mucosa or other respiratory tissue. Only two studies have identified the presence of AP in the respiratory cells. Bourne (1948) found AP in human nasal mucosa and it has also been found in the nasal epithelium of guinea pigs (Gawin et al., 1991). Conversely, in a histochemistry study involved isolating enzymes in the nasal mucosa, Shapiro (1968) showed that in embryonic stage of the rodents, only basal portions of olfactory epithelium stained positively for AP which was not present in other respiratory cells. Staining became more intense in older embryos, but continued to be localized in the basal one-third to one half of the olfactory epithelium. He concluded that the presence of this hydrolytic enzyme suggested AP involvement in the mechanism of olfaction.

From this histochemistry study it was apparent that the NSAP was produced locally. The author believed that the discovery of nasal mucosa as the production source of bovine NSAP is a novel finding in research on AP. Previously nasal mucosa unlike liver, bone and intestine was not identified as the site of AP production in mammals. Although the source of NSAP have been successfully localised in the nasal mucosa, the precise mRNA location of this protein in the respiratory cells and the actual enzyme production pathway are yet to be studied and determined. Future work in this area should employ *in situ* hybridisation to define those matters. Unfortunately, due to lack of time and resources, the investigation of GGT that was also showing high activity in the bovine NS was not performed. It would be of great interest to determine whether GGT in NS is also produced locally by the respiratory cells.

Another notable finding in this chapter is that the distribution of AP activity in the cells of nasal epithelium and mucosa. Following the discovery that the NSAP was produced by the cells of the nasal mucosa, an objective in Chapter 3 was to measure and compare the AP activity from nasal mucosa to bovine tissue known to produce this enzyme, looking mainly at the liver, intestine and kidney. The AP extraction method used was similar to a method described by Rudolph et al. (1997) and result shown in activities per gram of wet tissue used. The AP derived from the nasal mucosa extracts (n=6) have a highest mean of activity followed by liver, kidney and extracts from intestinal mucosa, but this findings was not statistically difference. However, all of these extracts had significantly higher AP activities compared with the heart tissue that served as a negative control. It was concluded that although the AP activities between nasal

mucosa and AP producing tissues were not significantly difference, AP was still actively produced and released by the nasal mucosa which justify further investigation on the role and purpose of AP present in the bovine NS. Comparison of AP activity with bone extracts was not included in this study due to the lack of fresh bone sample. It would be important to include bone tissue in future study of AP activity due to its vital role in calcium homeostasis and during skeletal mineralization.

In order to detect AP protein and quantify the concentration of AP in nasal tissue, western blot analysis was performed. The antibody used in this study was a monoclonal antibody raise in mouse against human bone AP (abcam 17272) and was conjugated with HRP using a commercial conjugation kit. However, although it was a monoclonal antibody, the western blot with anti AP revealed four visible protein bands at approximately 30, 55, 70 and 150 kDa in nasal mucosa and liver extractions (Figure 3.4). Further western blot analysis to determine the specificity of antibody used was carried out in NS and in various tissues extracts including BSA as a negative control but have resulted the antibody binds with bovine albumin at approximately 70 kDa. The findings were discussed in detail in section 3.4 of this thesis. It was concluded that the antibody used in this study was not specific in detecting AP protein hence restricting further analysis and quantification of the AP concentration in nasal mucosa and other related tissues. Further study with antisera specific for bovine AP from NS is warranted.

An initial attempt to stain for AP following native PAGE was not successful, possibly the AP did not enter the gel without the reduction that usually is involved in SDS-PAGE. A study for AP separation using agarose gel electrophoresis was undertaken to compare the electrophoretic mobility of bovine AP isoenzymes from NS and nasal mucosa with other bovine tissues. After stained with AP specific substrate, results have shown that AP from NS and nasal extracts has the same size and is clearly travelled slower than the liver extract (Figure 3.9). However, the AP bands from other tissue extracts (bone, kidney and intestine) show no clear difference in electrophoretic mobility with NS and nasal mucosa. Therefore, AP extracts from the various bovine tissues were subjected for separation by IEF according to pIs to aid in identification of relative isoenzymes.

Thus IEF was able to separate the AP and allowed the enzyme activity to be detected by AP activity staining. Although similar AP activity was loaded on to the IEF gel (Figure 3.10), inspection revealed notable differences in the band intensities. This difference was possibly due to differences in the ability of the AP enzyme to penetrate the polyacrylamide gel which was especially evident in the NS samples. This could be due to

the formation of aggregates, possibly with high molecular weight and complex proteoglycans contained in nasal mucus remaining at the sample well. Smearing was also seen on one of the IEF track from NS sample and its greater activity compared to sample the other NS sample may be due to partial degradation of AP molecules during sample preparation which could allow greater penetration of the gel by smaller AP forms.

Staining was carried out at pH 9.2 and as there is a possibility that different AP isoenzyme would optimally stain at different pHs and this may also contribute to the variation seen in AP activity on the IEF gel. Nevertheless, the AP isoenzyme in NS had pIs in the same region as those of the nasal mucosa extracts (pH 4.2-6.8) but was clearly different from the extracts of other tissues. The differences in IEF migration of the AP extracts is likely to be due to PTMs such as glycosylation or phosphorylation, the elucidation of which would be the subject of further investigation.

Chapter 4 investigated assessed the genomic part of nasal AP using advance molecular methods; endpoint RT-PCR to detect the AP gene expression in nasal tissue and gene sequencing to compare its nucleotides with other tissues known to produce AP mainly from the liver and intestine. Database and literature searches suggest that the only known gene that expressed AP in bovine tissue were the tissue non-specific AP gene (*ALPL*; NM_176858) and intestinal AP gene (*ALPI*; NM_173987). Preliminary work successfully generated primer sequences (Table 4.1) to differentiate between the bovine *ALPL* and *ALPI* genes for the amplication of bovine AP present in nasal, liver and intestinal tissues. The analysis at mRNA levels from nasal mucosa by endpoint RT-PCR and PCR product sequencing confirmed that the AP was locally produced and has homology at the nucleotide level to the tissue non-specific AP splice variant gene found in bovine liver, bone and kidney. Another potential study that would be worthwhile is to quantify the nasal AP gene expression by qPCR which would be significant in determining the rate of expression in nasal mucosa and comparison with other relevant AP producing tissues and assess changes during BRD.

The specific reason and precise location in the respiratory cells that express the mRNA encoding tissue non-specific AP genes within the nasal tissues is unclear, but the induced expression probably results from the reaction of certain cytokines or hormones with their cellular receptors in response to invading pathogens and inflammatory signals. The difference in pIs that has been demonstrated in Chapter 3 strongly suggests PTMs of the nasal derived AP hence increasing the functional diversity of the enzyme.

In addition, although non-specific AP and IAP were expressed by different gene, both of the AP genes encode enzymes with well conserved catalytic activities hence is possible to have the ability to dephosphorylate LPS endotoxin as was described in detail in section 1.3.4.

Apart from the reported ability of IAP to dephosphorylate LPS, Picher et al. (2003) have suggested that AP present within the respiratory cell is capable to dephosphorylate ATP to AMP and to adenosine, a molecule that is important for maintaining mucociliary clearance rate. Furthermore in this species, NS is commonly transferred to the mouth as cattle have the tendency to lick their muzzle and nostrils so that the nasal AP could be effective in the rumen as an anti-endotoxin with a dephosphorylation activity (Lalles, 2014). Although it was speculated and liken the nasal AP function with the ability of IAP to dephosphorylate LPS, the definitive role of nasal AP and how it participates in the nasal innate response could be a very important and interesting area to explore in the future and definitely beneficial to the diagnosis and control of BRD.

The research was also driven by the rapid advancement and the easy accessibility of the various proteomic technologies. The main objective in Chapter 5 was to identify the proteome of healthy bovine NS in the awareness that it is vital to define a “normal” profile of NS proteins so that the usual function of this secretion can be well-determined and disease-specific changes can be identified. For this chapter, conventional 1-DE was used to conduct a preliminary investigation on the protein contents in NS. Prior to that, the protein concentrations of 38 NS samples collected from healthy cattle were determined resulted with a mean of 15±6.7 g/L (±SD). On 1-DE a total of 13 distinct protein bands were visible after NS protein was separated according to Mw.

Initial application of 2-DE on six individual NS samples showed a similar spot pattern with little variation observable. Subsequently 2-DE of pooled NS (n=6) produced a gel with well separated protein spots. Thirty apparent protein spots were selected and process for identification. Protein identification by using ESI-MS/MS analysis on proteins bands from 1-DE and spots from 2-DE have identified similar proteins within the gels consist of vascular derived proteins involve mainly in regulatory and innate immunity and also glandular derived proteins like OBP and lactoferrin. Interestingly, glutathione-S-transferase an enzyme capable of detoxifying noxious compounds were detected only in 2-DE and was not discovered by other means of protein separation used in this study. The proteomic findings on NS were collected later served as a baseline parameter of normal bovine NS for comparative proteomic study conducted in Chapter 6.

However, although the 2-DE method used in this study successfully isolated and allowed identification of protein expression, a series of limitations have to be dealt with when using this proteomic approach. For instance preparation of 2-DE needs intensive work and not all proteins can be resolved; membrane proteins are not represented because of their poor solubility, and low abundant proteins cannot be detected because they are often lost during the electrophoretic procedure (Gygi and Aebersold, 2000 and Santoni et al., 2000). In addition, 2-DE also limits the assessment of more than a group of sample and analyses of the changes in proteins expression. Therefore, these issues need to be solved in order to ensure appropriate comparison between groups and, thus, advance identification of any possible markers to differentiate between disease and healthy animals.

Even though 2-DE results can be variable among gels, an accurate comparison requires first of all that protein samples are separated via the same electrophoretic system to ensure consistency. The system of two-dimensional fluorescence difference gel electrophoresis (DiGE) is another variant of 2-DE and would offer the possibility of including an internal standard making comparison of gels easier and quantification of proteins more accurate (Marouga et al., 2005).

Chapter 6 aimed to use DiGE as an advance comparative proteomics tool to detail the changes in the NS protein profiles from healthy, disease and vaccinated group of animals. Nasal secretion used in this study derived from an earlier immunisation study against bovine malignant catarrhal fever (MCF; Haig et al., 2008). The selection of NS for the disease and vaccinated group were described in detail in section 6.2.6. In general, animal given vaccination and booster by intramuscular (IM) and later challenged via intranasal route were selected as the ‘vaccinated group’ and those challenged without given any protection were selected as the ‘disease group’.

Difference gel electrophoresis has been used with success to find differentially expressed proteins in a number of studies with the major advantage of this technique being that it allows the comparison of two protein samples on the same gel. This method proved to allow for a more accurate detection of small differences in protein levels between samples (Alban et al., 2003). The high sensitivity of this method have enabled the detection of more than 500 protein spots per gel and the comparison between the 3 different group of NS have identified 23 proteins that were differentially expressed. The majority of the NS proteins identified in this study were akin to those identified in human NS (Casado et al., 2005) which mainly consists of proteins involved in innate and acquired immunity system. These have proved that bovine NS to have a

critical role in preventing microbial infections and protecting the LRT from any pollutants.

The most abundant plasma proteins (vascular derived proteins) in NS were albumin and immunoglobulins which accounts for more than half of all the protein that were differentially expressed between the 3 different groups. Most of the plasma proteins were down regulated in disease and vaccinated group. This is probably proteins such as fibrinogen and albumin that were actively involved in innate immune processes by forming a coagulated mass of mucins (Lindahl et al., 2001 and Ghafouri et al., 2002).

However, proteins such as serotransferrin, cathelicidin and complement factor C3 which are important for bacterial inactivation and immune complex formation decreased in expression whereas in the vaccinated group were observe to be highly expressed. The difference of expression was possibly due to the ability of host immune system to fully respond to vaccination hence providing protection to the host prior to challenge. Cellular proteins like lactoferrin, an antimicrobial protein commonly present in mucosal secretions were also down regulated in disease and increased in expression within the vaccinated groups. This situation might relate to its antiviral activity by actively being secreted and binds lipoproteins of cell membranes hence preventing the virus to penetrate into the cells (Nozaki et al., 2003).

On the other hand, immunoglobulins were up regulated in the disease and vaccinated group possibly as an acquired immune response to the intranasal challenge which could actively infiltrate from serum into the NS through an increase in vascular permeability during inflammation (Casado et al., 2005). In addition, another high abundance plasma-derived protein that is up regulated is the α-2-macroglobulin. This very high molecular weight protein is capable of passing through the molecular weight sieve created by the nasal epithelial basement membrane and together with α-2-microglobulin and ß-2-microglobulin forms the MHC class I complex (Svensson et al., 1995).

The function of OBP, apart being known to be involved in scent recognition, is also likely involves in protecting airways from lipid peroxidation (Grolli et al., 2006). However in this study, the increase in expression in disease and vaccinated group were still not clearly understood. Mitchell et al. (2011) have shown in immunochemistry that OBP was produced locally within the nasal mucosal glands and also show high expression in the tracheal and bronchial mucosal glands. However, Mitchell also reported that OBP does not become involved in host defence mechanism or alter bacterial growth. Previous work from the same group reported increased levels of OBP in the lung of

dexamethasone-treated calves suggesting that this may be one mechanism by which corticosteroids modulate pulmonary inflammatory responses, which implicates OBP as a moderator of inflammation that may influence the outcome of many pulmonary disease conditions (Mitchell et al., 2007).

In summary, Chapter 6 concluded that DiGE analysis has been able to detect several NS proteins which were differentially expressed in cattle under disease and vaccinated condition. Nevertheless the inherent drawbacks of DiGE that is present including limited sample capacity, insufficient detection of very acidic and basic proteins as well as hydrophobic membrane proteins and low visualization sensitivity were considered and look upon (Monteoliva and Albar, 2004). The application of a non-gel based technique could be a productive alternative to overcome many 2-DE limitations.

Shotgun proteomics (bottom-up strategy) is used to identify highly complex peptide fractions, generated after protein proteolytic digestion. This method is able to resolve peptides using different fractionation strategies, which offer high-throughput analyses of the sample proteome and provide a snapshot of the major protein constituents (Abdallah et al., 2012). This technology is also able to identify low-abundance proteins as well as highly acidic and basic proteins (Washburn et al., 2001). However, most of the shotgun methodologies are non-quantitative hence for quantification of potential biomarkers and assessment of protein expression between groups, quantitative methods based on protein labelling (such as SILAC and iTRAQ) or on unlabelled approaches to increase the coverage of all differentially expressed proteins present in NS should be considered for future studies.

There is a vital need for the discovery of markers that will detect BRD at an earlier stage. The discovery of potential biomarkers like AP in bovine NS would be a very desirable achievement due to the ease with which this secretion can be collected and has now been biochemically and molecularly characterized for future reference. All of the work detailed within this thesis strives towards this similar goal. The emergence of new methodologies for the large-scale analysis of differentially expressed proteins in body fluids is presenting opportunities in the search for novel diagnostic markers of disease. The development and application of proteomic technologies with improved sensitivity could be applied to identify potential biomarkers of respiratory disease in NS, hence linking the major findings in this thesis.

7.1 Conclusion

In conclusion, the studies in this thesis have contributed significantly to the understanding of biochemical composition and protein expression in bovine NS and thus to the understanding possible mechanism by which NS is involved in bovine innate protection against respiratory diseases. The novel discovery of that AP is present in bovine NS is not fully understood which makes investigation of this secretion more challenging. Nevertheless, the establishment of a practical method of collecting NS and establishing its biochemical profiles enables studies of this fluid to be made in more depth, especially the more undiscovered areas including the biological function of nasal AP.

Additionally, the protein profiling as well as analysis of differentially expressed nasal proteins has provided information in the response of the animal under stress situation such as in post-vaccinated or disease state which has not been reported before. The proteins catalogued were also an important resource for the comparison of future proteomics studies involving NS as well as quantifying responsiveness to vaccine hence contributing to the control and prevention of respiratory diseases in cattle.

Appendices

Appendix A

Publication

MF Ghazali, HHC Koh-Tan, M McLaughlin, P Montague, NN Jonsson and PD Eckersall 'Alkaline phosphatase in nasal secretion of cattle: biochemical and molecular characterisation' *BMC Veterinary Research*. **2014**, 10 (1), 204.

Ghazali *et al. BMC Veterinary Research* 2014, **10**:204
http://www.biomedcentral.com/1746-6148/10/204

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Alkaline phosphatase in nasal secretion of cattle: biochemical and molecular characterisation

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Abstract

Background: Nasal secretion (NS) was investigated as a source of information regarding the mucosal and systemic immune status of cattle challenged by respiratory disease. A method for the collection of substantial volumes (~12 ml) of NS from cattle was developed to establish a reference range of analytes that are present in the NS of healthy cattle. Biochemical profiles of NS from a group of 38 healthy Holstein-Friesian cows revealed high alkaline phosphatase (AP) activity of up to 2392 IU/L. The character and source of the high activity of AP in bovine NS was investigated.

Results: Histochemical analysis confirmed the localization of the AP enzyme activity to epithelial cells and serous glands of the nasal respiratory mucosa. Analysis of mRNA levels from nasal mucosa by end point RT-PCR and PCR product sequencing confirmed that the AP was locally produced and is identical at the nucleotide level to the non-specific AP splice variant found in bovine liver, bone and kidney. Analysis by isoelectric focussing confirmed that AP was produced locally at a high level in nasal epithelium demonstrating that AP from nasal secretion and nasal mucosa had similar pI bands, though differing from those of the liver, kidney, bone and intestine, suggesting different post-translational modification (PTM) of AP in these tissues.

Conclusions: A nasal isozyme of AP has been identified that is present at a high activity in NS, resulting from local production and showing distinctive PTM and may be active in NS as an anti-endotoxin mediator.

Keywords: Bovine, Nasal secretion, Alkaline phosphatase, Isoelectric focusing, RT-PCR

Background

Bovine respiratory disease (BRD) is an important economic challenge to beef production globally and is estimated to cost the UK cattle industry £60 million annually [1]. We wished to investigate bovine nasal secretion (NS) as a source of information regarding the mucosal and systemic immune status of cattle in health and in relation to BRD.

To use a body fluid such as NS for detection and monitoring of disease it was necessary to establish a reference range of analytes that are present in the NS of healthy cattle. However, for bovine NS there is no information on the composition of the secretion, possibly due to the perceived difficulty of collecting a sufficient volume of NS for analysis. A method was therefore developed which allowed collection of over 10 ml of NS, sufficient for multiple investigations. Thereafter in the course of biochemical analysis of NS, using a panel of tests that are used in veterinary clinical biochemistry analysis, it was found that the activity of the enzyme, alkaline phosphatase (AP; EC 3.1.3.1) in the NS was markedly higher (up to 2392 IU/L) than the activity found in healthy bovine serum (20–80 IU/L).

In cattle, as in other animals, AP exists as several isozymes in different tissues, encoded by two known genes: one for the intestinal form (IAP) and the other for the non-specific AP which occurs in bone (BAP), liver (LAP) and placenta (PLAAP) [2-4]. A major biochemical function of the enzyme family is to dephosphorylate molecules with consequent change in function of those molecules. Recent investigation into the function of AP has revealed actions that could be relevant to its presence in NS. One of these is the dephosphorylation of bacterial lipopolysaccharides (LPS) resulting in detoxification of the LPS [5-8].

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For IAP this has been shown to have a role in the maintenance of gut homeostasis, favouring the proliferation of commensal bacteria over pathogens such as *Salmonella typhimurium* [9]. This activity is proposed to be related to local immunomodulating effects, likely via regulation of the LPS-toll-like receptor 4 (TLR4) interactions between gut microflora and intestinal epithelium [10].

Within the respiratory tract, AP activity has been previously demonstrated within the cell and on the surface of respiratory epithelium in humans, where it is able to dephosphorylate ATP to AMP and to adenosine, important for mucociliary clearance [11]. The enzyme was also reported several decades ago in human nasal mucosa [12] and more recently described in this tissue and, though in micro litre volumes, of NS of guinea pigs [13]. Alkaline phosphatase activity has also been found in the olfactory epithelium of mice and rats [14]. Apart from these reports, the activity and production of AP in NS and nasal mucosa has not, to our knowledge, been documented. The ability to collect substantial (ml) volumes of bovine NS has enabled a biochemical analysis of the NS and investigation of the possibility that the high activity of AP in NS has a significant biological function.

The AP in NS could originate from either local synthesis or secretion from cells in the bovine nasal epithelium or it could be a result of leakage from serum. However the latter theory is not supported by the finding of higher activity of AP in NS than in serum unless a mechanism to export the AP against a concentration gradient is present in this tissue. The objective of this investigation was to characterise and identify the source of the AP in bovine NS and establish if this AP has similarity to established isoforms of the enzyme.

Methods

Animals and collection of nasal secretion

38 Holstein-Friesian cows aged 2–5 years were sampled from a herd of 90 lactating cows from University of Glasgow Cochno Farm. The animal experiments were carried out with the approval of the University of Glasgow MVLS College Ethics Committee and complied fully with the Home Office of Great Britain and Northern Ireland "Animals (Scientific Procedures) Act 1986". Clinical examination was performed to ensure the cows were clinically healthy and free from respiratory disease. The cows were restrained in a cattle crush during the procedure. A commercially available tampon was inserted into one nostril and slid gently in an upwards and backwards direction about 5–8 cm deep and then left in place for up to 15 minutes [15]. The tampon was then removed from the nostril by gently pulling on the attached string and weighed, as a measure of the NS uptake before being inserted into a modified collecting tube (Figure 1). The cows were observed during collection to ensure that there were no signs of discomfort but no signs of discomfort were observed at any time in any cow in the study. The modified tubes were centrifuged at 700 g for 10 minutes at 4°C in a procedure similar to that of Lu and Esch [16]. Nasal secretion collected at the bottom of Falcon tube was transferred into 1.5 ml tubes and stored at −80°C until further analysis.

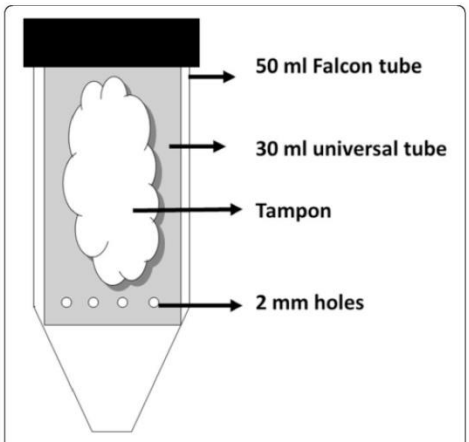

Figure 1 Schematic representation of a collecting tube for nasal secretion. The saturated tampon from nostril was inserted into a modified collecting tube consisted of a 30 ml universal tube with four × 2 mm holes drilled into the bottom, inserted in a 50 ml Falcon tube.

Protein extraction for biochemical and immunological analysis

Nasal mucosa, small intestine, heart, liver and kidney samples from 6 adult cattle were collected at a slaughterhouse and processed within 24–48 hours. Nasal mucosa was gently cut and separated from the nasal conchae using a forceps and blade. Intestinal mucosa was obtained by gently scraping the mucosa of the small intestine with a metal spatula. Tissues were snap-frozen in liquid nitrogen and stored at −80°C prior to extraction. Two grams from each tissue were washed with isotonic saline solution and homogenized (Janke & Kunkel Ultra Turrax T25) in 5 ml saline. AP was extracted from 2 samples of crushed cancellous bone obtained from the metacarpal bone of 2 cattle.

Tissue AP extracts were further processed by incubation with 100% n-butanol in ratio of 1:4 of n-butanol: protein homogenates, with gentle agitation at 4°C for 30 minutes [17]. Extraction with n-butanol facilitates the release of AP from membranes where it is anchored to phosphatidylinositol [18]. The samples were centrifuged at 1,500 g for 30 minutes at 4°C, the aqueous layer removed and centrifuged at 15,000 g for 90 minutes with

the supernatant being the AP extract. The AP activity and the protein concentration in the AP extracts were determined as described below. The AP activity of tissue extracts was recorded as enzyme activity per gram of tissue extracted (U/g). The relative AP activity in the different extracts was compared by a Kruskal-Wallis test (GraphPad Prism 5.0, California, USA) with a significance level (α) set at 0.05.

Biochemical and immunological analysis

The nasal secretions and tissue extracts were examined for biochemical composition using an Olympus A640 analyser operated by Veterinary Diagnostic Services, University of Glasgow. Enzymatic test were carried out at 37°C where measurement of AP were based on the recommendations of the German Society for Clinical Chemistry (GSCC) at a pH of 10.4. Analysis method of γ – glutamyl transferase (GGT) and aspartate transaminase (AST) were based on the recommendation of the International Federation of Clinical Chemistry (IFCC). Albumin was measured by a bromcresol green (BCG) binding assay, total protein by reaction with biuret, urea by reaction with 2-oxogluturate and nicotinamide adenine dinucleotide (NADH), creatinine by reaction with picric acid, total bilirubin by reaction with 3,5-dichlorophenyldiazonium tetrafluoroborate (DPD), calcium by reaction with o-Cresolphthalein-complexone (oCPC) and phosphate by reaction with molybdate. Measurements of electrolytes were carried out by ion-specific electrodes using an indirect potentiometry method. Measurement of bovine IgA and IgG concentrations using species-specific ELISAs (Bethyl Labs, Texas, USA) according to the manufacturer's instructions.

Histochemical analysis

Bovine nasal mucosa were collected for histology examination as described above with a 1 cm square dissected out and snap frozen in liquid nitrogen. Cryosections of 8 μm were fixed with acetone, stained with Vector® Red Alkaline Phosphatase Substrate Kit (Vector Labs, Peterborough, UK), in the presence or absence of levamisole inhibitor (Sigma-Chem Co, Poole, UK) counterstained with Gills Haematoxylin (Sigma-Chem Co, Poole, UK) and mounted with Histomount (National Diagnostics, New Jersey, USA). Images were taken with Olympus BX51 and attached DP71 camera (Olympus, Japan), using Cell^D software (Olympus, Japan).

Total cellular RNA extraction

Tissue samples of 100 mg from the same cattle used for the preparation of tissue extracts were used for RNA preparation using PureZOL™ (Bio-Rad Laboratories, Hemel Hempstead, UK) according to the manufacturer's protocol.

End point reverse transcriptase – polymerase chain reaction (RT-PCR)

Reverse transcription (RT) reactions were performed on RNA samples from nasal mucosa, liver and small intestine. 2 μg RNA samples were denatured at 65°C for 5 minutes. 50 μl reactions comprising 2 μg of total RNA sample, 1 × First strand buffer, 0.5 mM each of dNTPs, 10 mM dithiothreitol, 3 μg random primers, 20 U of RNAseOut and 400 U M-MVL reverse transcriptase (Life Technologies Ltd, Paisley, UK) were incubated for 30 minutes at 37°C, 60 minutes at 42°C, 15 minutes at 70°C and quenched at 4°C. The PCR products were analysed on 2% (w/v) agarose gels containing ethidium bromide (0.5 μg/ml).

Primers were designed to amplify specific regions of the bovine intestinal AP (IAP) gene ALPI (NM_173987) and non-specific AP gene ALPL (NM_176858), respectively using an interactive web-based primer program algorithm, GeneFisher software version 1.2.2 (BiBiServe, Bielefeld, Germany), with a predicted size PCR product of 500 bp. The primer sequences are depicted in Table 1. A total volume of 25 μl PCR reaction was prepared, comprising 12.5 μl RedTaq® DNA polymerase buffer (Sigma Chem. Co, Poole, UK), 0.5 μl of each primer (5 pmol per reaction tube), and 200 ng gDNA. Amplification conditions were 30 cycles (94°C for five minutes, 94°C for one minute, 58°C for one minute, 72°C for one minute, 72°C for 10 minutes). The PCR products (2 μl) were visualised using ethidium bromide (0.5 μg/ml) stained agarose gel and quantified against a 100 bp mass ladder for DNA (Life Technologies, Paisley, UK).

Sequencing

PCR products from RT-PCR reactions were purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The PCR products were sequenced using Big Dye v3.1 Terminator (Life Technologies, Paisley, UK) in 10 μl reaction volume containing 200–500 ng template, 1X sequencing buffer, 32 mM primer and 1x Big Dye mix. The sequencing reactions were carried out in two random nasal mucosa samples with forward and reverse primers in separate reactions. The sequencing products were ethanol precipitated, resuspended in formamide and read using capillary

Table 1 Primer sequences used for RT-PCR

Gene	Accession number	Primer notation	Sequence 5'-3'
ALPI	NM_173987	ALPI-F	GGGAGTGGTGACCACCTCCA
		ALPI-R	GTCAATGCGGCCTCCCTCCA
ALPL	NM_176858	ALPL-F	GACAGCTGCCCGCATCCTCA
		ALPL-R	CCTTCTCATCCAGCTCATACTCCA

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electrophoresis on Applied Biosystems 3130XL Genetic Analyzer (Hitachi, Tokyo, Japan).

Isoelectric focusing (IEF)

The pI of the various AP isozymes was determined by separation using Invitrogen Novex® pH 3–7 IEF Gel (Life Technologies, Paisley, UK). Samples of bovine NS and tissue extracts from NS, nasal mucosa, small intestine, liver, kidney and bone were selected at random from the available samples for the separation of AP isozymes. Samples were diluted to an AP activity of 300–350 IU/L in IEF sample buffer pH 3–7 (Life Technologies, Paisley, UK) prepared according to manufacturer's instructions. Ten microliters of the prepared sample were loaded into each well such that equal activities of AP were loaded into each well. Isoelectric focussing was conducted at 4°C using the following voltage gradient: 100 V, 1 hour; 200 V, 1 hour; 500 V, 30 minutes. The gel was then stained with Pierce 1-Step™ NBT-BCIP ALP substrate solutions at pH 9.2 (Thermo Fisher Scientific Inc, Illinois, USA) for 5 hours at 37°C. The pI of focussed bands was estimated in comparison to pH 3–10 standards (Serva Electrophoresis, Heidelberg, Germany) which were run in a track of the IEF gels, with the track being excised and stained separately with Coomassie blue following isoelectric focusing.

Results

Biochemistry of bovine nasal secretion

A volume of between 5–12 ml of NS was collected from each of 38 Holstein-Friesian dairy cows. Protein concentrations in nasal secretion ranged from 9 to 34 g/L. Following biochemical analysis the mean (±SD) AP activity in NS was 1239 ± 553 IU/L and was up to 16-fold higher than the serum reference range for AP (Table 2) while GGT activity was 2.6-fold higher than the bovine serum GGT reference range. Concentrations of total protein, albumin, calcium, phosphate, sodium and chloride were lower than the serum reference range while urea, creatinine, bilirubin and aspartate transaminase levels were comparable to serum reference ranges. The concentrations of IgA and IgG were 0.5 - 2 g/L and 0.2 - 1.9 g/L respectively.

Distribution and localisation of AP activity

The extracts from nasal mucosal tissue (n = 6) had mean AP activities which were not significantly different from the mean activity extracted from other bovine tissues known to produce this enzyme: liver, intestine and kidney. All of these extracts had significantly higher AP activities per gram of wet tissue extracted compared with the heart tissue (Table 3) that served as a negative control.

Table 2 Biochemical and immunological compositions of bovine nasal secretion (n = 38)

Analytes (unit)	Median (Range)	Reference range (Bovine serum)[a]
Urea (mmol/L)	3.1 (1.8 - 6.1)	0 - 8
Calcium (mmol/L)	1.1 (0.6 - 1.4)	2.2 - 3.3
Phosphate (mmol/L)	0.8 (0.4 - 1.6)	1.1 - 2.8
Creatinine (μmol/L)	30 (5 - 61)	53 - 132
Total bilirubin (μmol/L)	0 (0 - 2)	0 - 8
Total protein (g/L)	14 (9 - 34)	52 - 84
Albumin (g/L)	3 (0 - 5)	21 - 34
Sodium (mmol/L)	60 (18 - 98)	135 - 151
Potassium (mmol/L)	2.4 (0.6 - 3.9)	3.2 - 5.8
Chloride (mmol/L)	19 (14 - 34)	96 - 111
Alkaline phosphatase (IU/L)	1155 (144 - 2392)	20 - 80
Aspartate transaminase (IU/L)	97 (50 - 184)	10 - 140
Gamma-glutamyltransferase (IU/L)	68 (28 - 109)	0 - 27
IgA (g/L)	1.2 (0.5 - 2.0)	1.8 - 3.9[b]
IgG (g/L)	0.5 (0.2 - 1.9)	0.1 - 0.7[c]

[a]Laboratory reference range for bovine serum; [b]Morgan et al., [19]; [c]Duncan et al., [20].

Histochemistry using an AP activity stain showed strong AP activity (Figure 2) in the luminal and basal ends of nasal epithelium (yellow arrow) and serous glands (green arrow). AP activity staining was abolished in the presence of levamisole a known non-specific AP inhibitor.

Analysis of nasal mucosa AP mRNA abundance levels

A single PCR product at the predicted size of 500 bp was detected in the nasal mucosa using primers for bovine non-specific AP gene ALPL (NM_176858) (Figure 3a) but not with primers for the intestinal AP (IAP) gene ALPI (NM_173987) (Figure 3b). Liver and intestine cDNAs served as positive and negative controls for each primer pair. Sequencing of PCR products from two biological samples of nasal tissue showed homology to the ALPL splice variant.

Isoelectric focusing of AP isoforms

The AP from various bovine tissues separated by IEF and stained by zymography with an AP susbstrate, are

Table 3 AP activity in bovine tissue extracts (n = 6)

Tissue	Median (IU/g) (Range)
Nasal mucosa	14.3 (1.0 - 27.6)
Intestinal mucosa	6.5 (2.2 - 24.0)
Heart	0.6 (0.2 - 1.1)*
Kidney	7.4 (3.9 - 16.2)
Liver	8.3 (1.0 - 27.2)

*AP activity was significantly different from all other tissue extracts ($P < 0.05$).

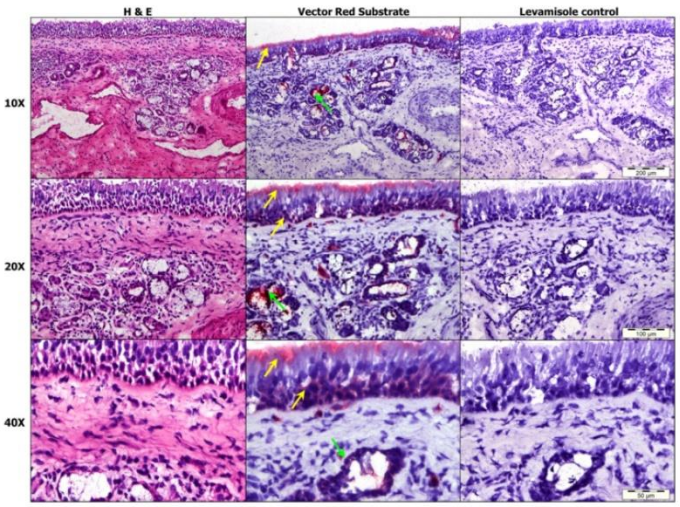

Figure 2 Histology and alkaline phosphatase activity histochemistry of nasal mucosa. Routine staining of nasal mucosa with Haematoxilin and Eosin (left column) and histochemical staining of AP activity using Vector Red substrate (central column) showed strong AP activity in the mucus at the luminal surface and in the basal cell layers of the epithelium (yellow arrow); as well as in serous glands (green arrow). Levamisole, a non-specific AP inhibitor, abolished the histochemical staining of these cells (right column). Image captures at x10, x20 and x40 magnifications are shown with appropriate scale bars.

shown in Figure 4 including boxes around selected results. The IEF demonstrated that AP from the NS (run in duplicate) had isoforms forming bands of AP activity with pI of 4.8 (Figure 4–1) and 5.0-5.3 (Figure 4–2). There were inter-individual difference found in the AP bands from NS samples at pI 5.0-5.3 illustrated by NS samples (a) and (b) with sample (b) showing greater activity and had a smeared appearance. The nasal mucosal extract also had major bands with pI 4.8-5.2 (Figure 4–3) and several minor bands with pI of 5.3 - 6.2 (Figure 4–4). Bovine bone AP had an isoform with pI of 4.8 (Figure 4–5) and 5.0-5.3 (Figure 4–6), with several bands with pI of 6.0 – 6.7 (Figure 4–7). The liver AP focussed with lower pI showing a major band at pI 4.6 (Figure 4–8). Intestinal and kidney AP had isoforms with pI of 5.4-6.2 (Figure 4–9) while kidney AP also having major bands between with pI of 5.2 and 5.3 (Figure 4–10). The pI of the isoforms were estimated by comparison to standard protein of known pI value run on the same IEF gel with the track of the standard proteins separated and visualised with Coomassie blue protein stain rather than with AP zymography. Although diluted to a similar AP activity, notable differences were observed in the band intensity of the AP isoforms. Close inspection of the loading

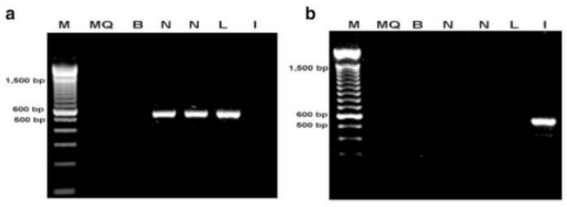

Figure 3 Alkaline phosphatase mRNA analysis from bovine tissues by RT-PCR. Bovine tissues examined by RT-PCR to determine expression of genes for AP. The presence of a single 500 bp DNA fragment corresponding to the predicted product size was visualised when using **(a)** non-specific AP primers and **(b)** intestinal AP primers. PCR products were present in nasal mucosa only when non-specific AP primers were used. M = Marker (100 bp DNA ladder); MQ = high purity water; B = blank; N = nasal mucosa; L = liver; I = intestine.

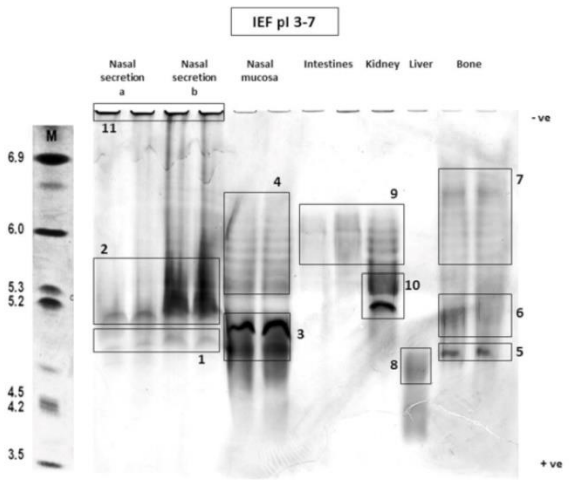

Figure 4 Isoelectric focussing of alkaline phosphatase in bovine tissue and nasal secretion. Bovine tissue extracts and nasal secretion separated on isoelectric focusing gels over a pH 3–7 range and stained with 5-Bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium chloride (BCIP/NBT) substrate. M = IEF standard protein marker (stained with Coomassie blue); –ve = cathode; +ve = anode. Two different samples of NS were run in duplicate. Samples of extracts from nasal mucosa, bone and intestine were run in duplicate with liver and kidney extract being run in single tracks. Annotated boxes around isoforms bands as discussed in the text.

well at the cathodal end of the gel, for the NS samples showed a deposit of AP activity, suggesting that a proportion of the AP in these samples were unable to enter the polyacrylamide gel, possibly due to the formation of large aggregates.

Discussion

This investigation had the original objective to collect and characterise bovine NS as a readily available biological fluid in the search for biomarkers of BRD. However prior to this, it was necessary to establish the normal components of the fluid and their concentration in NS from healthy cattle. Swabbing the nasal tract proved to be an inefficient method of collection of the fluid. A system was therefore developed which allowed bovine NS to be collected in sufficient volume for analysis. Previous studies have collected NS on tampons [15] but in this study the sample was removed from the absorbent by centrifugation using modified centrifuge and universal tubes, which allowed up to 12 ml of NS to be collected from each animal.

Biochemical analysis showed that most analytes were at similar or lower concentrations to those in bovine serum. Unexpectedly, it was found that the activities of AP and GGT were greater by factors of close to ten-fold and three-fold respectively in the NS than in serum from healthy animals. To our knowledge there are no previous reports of the activities of these enzymes in NS being greater than that found in serum or plasma. Indeed in the classic text on AP by McComb et al. [2] there is no mention of AP in nasal mucosa or NS and only one reference to AP in olfactory cells related to the nasal cavity [21]. The lack of prior reports on the activity of AP in NS is likely to be due to the difficulty of collection of sufficient volumes of this fluid from animals such as laboratory rodents and reluctance of human volunteers to undertake NS collection. Whether the high activity of GGT in NS is also produced locally is an open to question and would require confirmation by gene expression or by histochemical analysis.

Alkaline phosphatase is synthesised in several tissues in mammals and distinct gene products have been identified in cattle with one form being produced in the intestine (IAP) and a second isoform, non-specific AP, being synthesised in liver, kidney and bone [3,22]. Recently, a study conducted by Yang et al. [4] discussed the evolution of AP genes, indicating that mammals have lost 1 of 3 clades present in zebrafish, however they also indicated that IAP is regulated by Myd88 dependent innate immune signalling with activity in reducing host responses to inflammatory microbiota products [4]. An activity based AP specific histochemical stain was used on sections of bovine nasal epithelia and indicated that AP is present in the mucus layer and in secretory cells

of the nasal epithelium. Using a similar histochemical approach, the enzyme has been previously identified in human nasal mucosa [12] in the nasal epithelium of guinea pigs [13] and the olfactory epithelium of mice and rats [14] but only limited volumes of NS were available for analysis. The study of AP in the nasal epithelium of guinea pigs [13] did detect AP activity in NS collected by lavage, but only a total volume of 30 μl of lavage fluid was collected per guinea pig, which would not be a sufficient volume for many investigations. Measuring AP activity from nasal mucosa for comparison to extracts of other bovine tissues revealed that the activity of AP per gram of tissue from nasal mucosa was equivalent to that of liver, kidney and intestine indicating that nasal mucosa is a major site for AP synthesis and secretion.

Enzyme isoforms can be separated by electrophoretic means to aid in identification of relative isoforms. Isoelectric focusing was able to separate the AP and allowed the enzyme activity to be detected by AP activity staining. Although similar AP activity was loaded on to the IEF gel, inspection revealed notable differences in the band intensities. This difference was possibly due to differences in the ability of the AP enzyme to penetrate the polyacrylamide gel which was especially evident in the NS samples (Figure 4–11). This could be due to the formation of aggregates, possibly with high molecular weight and complex proteoglycans contained in nasal mucus remaining at the sample well. Smearing of IEF track from NS sample (b) (Figure 4–2) and its greater activity compared to sample (a) may be due to partial degradation of AP molecules during sample preparation which could allow greater penetration of the gel by smaller AP forms. Staining was carried out at pH 9.2 and as there is a possibility that different AP isoform would optimally stain at different pHs and this may also contribute to the variation seen in AP activity on the IEF gel. Nevertheless, the AP isoforms in NS had pIs in the same region as those of the nasal mucosa extracts (pH 4.2-6.8) but were clearly different from the extracts of other tissues. The differences in IEF migration of the AP extracts is likely to be due to post translational modifications (PTMs) such as glycosylation or phosphorylation, the elucidation of which would be the subject of further investigation.

PCR using primers based on the published sequences of bovine intestinal [4] and non-specific AP [3] established that the nasal mucosa PCR product is identical to the bovine non intestinal AP messenger and was not related to intestinal AP. This was further confirmed by sequencing the PCR product which showed complete sequence identity with the published bovine non-specific AP sequence [22].

Recent studies of the biological function of AP have revealed that the enzyme has the ability to dephosphorylate lipopolysaccharide (LPS) endotoxin from Gram negative bacteria [23-25] reducing the toxic effects of LPS [26] and, in the intestine, can be induced by Resolvin E1, an anti-inflammatory derivative of omega-3 fatty acids [27]. Thus intestinal AP has a major function in reducing the effects of LPS from intestinal bacterial flora [5] and may have a similar function in livestock [28]. If AP in NS has the same activity on LPS then its presence in this secretion could contribute to the host defences by acting against invading pathogens in the respiratory tract responsible for BRD. It is of interest that AP in bronchial and alveolar fluid is believed to function in the extracellular dephosphorylation of ATP to fuel the activity of cilia in the airways [11] while there may also be interaction between ATP and nasal AP in minimizing inflammation as has been suggested for IAP [8]. This may be relevant to its presence in NS, while de-phosphorylation of the proinflammatory nucleotide uridine diphosphate (UDP), an action of IAP [29] and the relative stability of UDP on the airway surface of human [30] could provide nasal AP as a potential anti-inflammatory action with therapeutic value for the treatment of airway diseases. Furthermore in this species, NS can be transferred to the mouth as cattle have the tendency to lick their muzzle and nostrils so that the nasal AP could be effective in the rumen as an anti-endotoxin with a de-phosphorylation activity [8].

Conclusion

This investigation has shown that AP is present in substantial activity in bovine NS. We have demonstrated that AP activity found in bovine NS is produced locally in the nasal epithelium and does not result from transfer from the serum. This is based on the findings that AP was extracted from nasal epithelium with a similar efficiency as from liver, bone, kidney or intestinal tissue, AP activity was detected by histochemistry in sections of the nasal epithelium and the mRNA for bovine non-specific AP was present in this tissue. Isoelectric focusing demonstrated differences between the AP of NS and extracts of AP from other tissues with differences likely to be resulting from PTMs. The discovery of AP in nasal epithelium and NS in cattle presents opportunities for further investigation of the role of the enzyme in the susceptibility of mammals to infection through the nasal route.

Competing interests
The authors declare they have no competing interests.

Authors' contributions
Experimental design and planning: MFG, PDE, NNJ; Sample collection and processing: MFG; Laboratory analysis: MFG, HHKTC, MM, PM: Interpretation of data: all authors; Drafting the manuscript: MFG, PDE, NNJ. All authors read and approved the manuscript.

Acknowledgements
The authors are grateful to the Ministry of Higher Education of the Government of Malaysia for funding (MFG). The Veterinary Diagnostic Service of the School of Veterinary Medicine, University of Glasgow is thanked for performing the biochemical analysis.

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Received: 13 May 2014 Accepted: 27 August 2014
Published online: 05 September 2014

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doi:10.1186/s12917-014-0204-9
Cite this article as: Ghazali *et al.*: Alkaline phosphatase in nasal secretion of cattle: biochemical and molecular characterisation. *BMC Veterinary Research* 2014 **10**:204.

Appendix 2.1 (section 2.2.3)

Bradford reagent (5X concentrate)

100 mg Coomassie Brilliant Blue G-250

47 ml Methanol (100%)

100 ml Phosphoric Acid (85%)

QS to 200 ml with H_2O

NOTE: Coomassie must be dissolved in the methanol first before the other ingredients are added.

Appendix 2.2 (section 2.2.4)

Albumin

A coloured complex is formed when bromocresol green (BCG) reacts with albumin. The absorbance of the albumin-BCG complex is measured bichromatically at 600/800 nm and is proportional to the albumin concentration in the sample.

Reaction principle:

$$\text{Albumin + BCG} \xrightarrow{\text{pH 4.2}} \text{Green complexes}$$

Calcium

Calcium ions react with o-Cresolphthalein-complexone (Ca-oCPC) in an alkaline medium to form a purple coloured complex. In this method the absorbance of the Ca-oCPC complexis measured bicromatically at 570/660 nm. The resulting increase in absorbance of the reaction mixture is directly proportional to the calcium concentration in the sample.

Reaction principle:

$$Ca^{2+} + \text{oCPC} \xrightarrow[\text{pH 10.6}]{\text{8-Hydroxyquinolone}} \text{Ca-oCPC Complex}^{2+}$$

Sodium, Potassium and Choloride

The Ion selective electrode (ISE) for sodium (Na^+), potassium (K^+) and chloride (Cl^-) employs crown ether membrane electrodes for Na^+ and K^+ and a molecular oriented PVC membrane for Cl^- that are specific for each ion of interest in the sample. An electrical

potential is developed according to the Nernst Equation for a specific ion. When compared to an internal reference, this electrical potential is translated into voltage and then into the ion concentration of the sample.

Creatinine

Creatinine forms a yellow-orange coloured compound with picric acid in an alkaline medium. The rate of change in absorbance at 520/800 nm is proportional to the creatinine in the sample.

Reaction principle:

Creatinine + picric acid $\longrightarrow$ Creatinine picrate complex

Phosphate

In plasma, serum and bodily fluids the majority of phosphate exists in the inorganic form (Pi), approximately 15% bound to protein and the remainder in complexes and free forms. Inorganic phosphate reacts with molybdate to form a heteropolyacid complex. The asorbance at 340/380 nm is directly proportional to the inorganic phosphorous concentration in the sample.

Reaction principle:

$$7\ H_3PO_4 + 12\ (Mo_7O_{24})^{6-} + 72\ H^+ \longrightarrow 7\ H_3PO_4(MoO_3)_{12} + 36\ H_2O$$

Total bilirubin

A stabilised diazonium salt, 3,5-dichlorophenyldiazonium tetrafluoroborate (DPD), reacts with conjugated bilirubin directly and with unconjugated bilirubin in the presence of an accelerator to form azobilirubin. The absorbance at 540 nm is proportional to the total bilirubin concentration. A separate sample blank is performed to reduce endogenous interference.

Reaction principle:

$$\text{Bilirubin} + \text{DPD} \xrightarrow[\text{Surfactant}]{\text{Caffeine}} \text{Azobilirubin}$$

Total Protein

Cupric acid in an alkaline solution react with proteins and polypeptides containing at least two peptide bonds to produce a violet coloured complex. The absorbance of the complex at 540/660 nm is directly proportional to the concentration of protein in the sample.

Reaction principle:

$$\text{Protein} + Cu^{2+} \xrightarrow{OH^-} \text{Blue violet complex}$$

Urea

Urea is hydrolysed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced in the first reaction combines with 2-oxoglutarate and nicotinamide adenine dinucleotide (NADH) in the presence of glutamate-dehydrogenase (GLDH) to yield glutamate and NAD^+. The decrease in NADH absorbance per unit time is proportional to the urea concentration.

Reaction principle:

$$\text{Urea} + 2\ H_2O \xrightarrow{\text{Urease}} 2\ NH_4^+ + CO_3^{2-}$$

$$\text{2-Oxoglutarate} + 2\ NH4+ + 2\ \text{NADH} \xrightarrow{\text{GLDH}} 2\ \text{L-Glutamate} + 2\ NAD^+ + 2\ H_2O$$

Aspartate transaminase

Aspartate transaminase (AST) catalyses the transamination of aspartate and 2-oxoglutarate, forming L-glutamate and oxaloacetate. The addition of pyridoxal phosphate to the reaction mixture ensures maximum catalytic activity of AST. The oxaloacetate is reduced to L-malate by malate dehydrogenase (MDH), while NADH is simultaneously converted to NAD^+. The decrease in absorbance due to the consumption of NADH is measured at 340 nm and is proportional to the AST activity in the sample. Endogenous pyruvate is removed by the LDH-reaction during the incubation period.

Reaction principle:

$$\text{2-Oxoglutarate} + \text{L-Aspartate} \xrightarrow{\text{AST}} \text{L-Glutamate} + \text{oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + NAD^+$$

Appendix 2.3 (section 2.2.6.1)

Coating buffer

0.05 M Carbonate-Bicarbonate

Adjust pH to 9.6 with 1M sodium hydroxide (NaOH)

ELISA wash and blocking solution

50 mM Tris

0.14 M NaCl

0.05% Tween 20

Adjust pH to 8.0 with 1M sodium hydroxide (NaOH)

Sample and conjugate diluent

50 mM Tris

0.14 M NaCl

0.05% Tween 20

Adjust pH to 8.0 with 1M sodium hydroxide (NaOH)

ELISA Stop Solution

0.18 M H_2SO_4

Appendix 2.4 (section 2.2.8.1)

SDS-PAGE Running Buffer

144 g Glycine

30.3 g Tris

10 g SDS

Add the glycine to 700ml of distilled water, once dissolved add Tris and SDS and finally made up the volume of 1L with dH_2O.

Appendix 3.1 (section 3.2.1.1)

10% buffered neutral formaldehyde

162.5	disodium hydrogen orthophosphate
87.5 g	sodium dihydrogen phosphate
2.5 L	37% formaldehyde.

Add dH_2O to give final volume of 25 L and adjust pH to 7.4 with 1M hydrochloric acid (HCl).

Appendix 3.2 (section 3.2.1.4)

70% absolute alcohol

140 ml	100% alcohol
60 ml	dH_2O

70% methylated spirit

70 ml	100% ethanol
30 ml	dH_2O

Appendix 3.3 (section 3.2.1.4)

1% acid alcohol

990 ml	70% ethanol
10 ml	Concentrated HCl

Appendix 3.4 (section 3.2.1.4)

Scott's tap water substitute

8.25 g	Sodium bicarbonate
50 g	Magnesium sulphate
2500 ml	Tap water
2 crystals	Thymol

Bring to a total volume of 2.5 L

Appendix 3.5 (section 3.2.1.5)

VECTOR® Red AP enzyme staining procedure

Step 1: Add 2 drops of Reagent 1 to 5 ml of 100 mM Tris-HCl, at pH 8.2 - 8.5 buffer*.

Step 2: Add 2 drops of Reagent 2 and mix well.

Step 3: Add 2 drops of Reagent 3 and mix well.

Step 4: Incubate tissue sections with the substrate at room temperature until suitable staining develops (generally 20-30 minutes provides good staining intensity).

Step 5: Wash sections in assay buffer for 5 minutes. Rinse in water.

* Make the working solution in 100 mM - 200 mM Tris-HCl, pH 8.2 - pH 8.5.

Appendix 3.6 (section 3.2.3.3)

Transfer buffer (1X)

6.06 g	Tris
28.8 g	Glycine
1600 ml	dH_2O
400 ml	Methanol

Mix well and adjust pH to 8.0 with 1M sodium hydroxide (NaOH)

Appendix 3.7 (section 3.2.3.3)

5% skimmed milk

5 g skim milk powder

100 ml 1X Tris-buffered saline containing Tween 20

Appendix 3.8 (section 3.2.3.3)

TTBS (10X)

60.2 g Tris-base

87.6 g Sodium Chloride (NaCl)

10 ml Tween 20

700 ml dh_2O.

Adjust pH to 7.4 with 1M hydrochloric acid (HCl) and make the volume up to 1L.

Appendix 3.9 (section 3.2.4)

2% Agarose gel

0.8 g Agarose powder (Sigma-Chem Co, Poole, UK)

40 ml TAE buffer (1X)

4 ml dH_2O

Dilute solution in microwave for 1-2 min then add 15 µl of Ethidium bromide after 30 sec.

TAE buffer (1X)

4.8 g Tris-base

1.1 ml Acetic acid

2 ml 0.5M EDTA

Adjust pH to 8.6 with 1M sodium hydroxide (NaOH) and make the volume up to 1L.

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