Study of the Genetics of the Porcine Acute Phase Proteins

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For the degree of DOCTOR OF PHILOSOPHY

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of
GLASGOW

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Oct. 2007

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Authors Declaration

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged.

Abigail Diack
October 2007

Abstract

The principle aims of the work presented in this thesis were to further investigate the porcine acute phase proteins (APP) and determine the factors influencing the baseline concentrations of haptoglobin (Hp), C-reactive protein (CRP), Pig major acute phase protein (Pig-MAP) and transthyretin (TTR).

The APP are used as markers of inflammation and sub-clinical disease and are considered potential biomarkers for pig health and welfare. They have also been identified as a possible means of breeding for disease resistance, however little is known about the genetics of the porcine APP. This study investigated associations between the APP genes and baseline concentrations and the heritability of those concentrations.

An enzyme linked immunosorbent assay (ELISA) was developed for the measurement of porcine CRP, in-house methods were used for the determination of Hp and TTR and a commercial assay was used in the measurement of Pig-MAP. A population of pure line high health boars (n=~345) from 7 lines was used in the initial study each of which had an archived DNA sample and serum samples available for use.

Baseline herd concentrations of the 4 APP were determined and correlations between Hp and CRP, Hp and Pig-Map and CRP and Pig-MAP were identified. Significant differences were found between the 7 breeding lines in CRP, Pig-MAP and TTR concentrations, indicating that selective breeding for performance traits may also have affected innate immune traits such as APP concentrations.

Single nucleotide polymorphisms (SNP) were identified in the 4 APP genes and 17 were genotyped across the boar population with line differences apparent in their allele frequencies for CRP, Pig-MAP and TTR. Statistical analysis showed that there were significant associations between 3 of the SNP located in the Hp gene and Hp baseline concentrations ($p<0.01$); all 3 SNP were also in high linkage disequilibrium. The association indicates that Hp is under genetic control and would also be better suited to use as a biomarker due to the lack of line effects in the boars.

A heritability study was carried out utilising a mixed sex population of 297 animals (120 male, 177 female) comprising 7 breeding lines located on 2 farms. Initial analysis identified significant differences between male and females in Hp concentration, between pig lines in Hp, CRP and TTR and between the 2 farm units for CRP concentrations. This study showed that the baseline concentrations of the 4 APP could be affected by a variety of factors such as sex, commercial line and individual farm units and this must be taken into account if they are to be used as biomarkers. Heritability was estimated at 0.15, 0.13, 0.12 and 0.07 for Hp, CRP, Pig-MAP and TTR, respectively. All 4 APP show low heritability in serum concentration, this may prove problematic if they are utilised as a breeding trait.

Overall, the findings from this study indicate that baseline concentrations of porcine Hp, CRP, Pig-MAP and TTR are influenced by various factors including sex, breeding line, and farm unit and this must be taken into account before they are utilised as biomarkers. They

are traits of low heritability but the evidence suggests there may be a genetic component to their regulation thus requiring further investigation.

Acknowledgements

It is with great pleasure that I thank and acknowledge the people who have helped and contributed to this thesis over the past three years. Firstly, I would like to start by thanking my primary supervisor Prof. P. D. Eckersall for sharing his knowledge and for his guidance throughout this project; it has been a privilege to learn from the grandfather of porcine APPs! Additionally, I would like to extend my sincere thanks to my co-supervisors; Prof. M. J. Stear who has patiently guided me through the world of genetics and statistics, Dr. M. A. Mellencamp for her help and industrial knowledge and finally Christy Gladney who has been a mentor, travel guide and a friend to me particularly in the final stages of this thesis.

I am especially grateful to the Genes and Proteins group; Dr. Laura Bence (assay queen!) and Dr. Fiona Campbell for all their technical help and for keeping me sane in the lab; Dr. Lisa Murphy, Chris McComb, Marge Robinson and Mary Waterston for technical and IT support and finally Kerry Rennie for showing me how not to do lab work, for being a friend and occasional drinking buddy.

I would also like to thank all the individuals based in Genus PIC, Franklin, Kentucky for looking after me for a summer, providing me with samples, showing me new techniques and teaching me to drive on the wrong side of the road. It was a wonderful experience and I am grateful for the opportunity.

I am indebted to various friends who have helped in their own unique ways; Roni Wright and Chris Seagull for sharing the postgrad office, the Genus PIC and Genesis-Faraday students (who would have thought pigs or hangovers could be so much fun) and Treehugger for his cheery outlook and being a computer god.

Finally, I would like to extend my gratitude to my three letter family for letting me join them. The past 2 years have been filled with much happiness and a great deal of this was due to their love and support.

The past three years have taught me a great deal and it has been an honour to meet and work with so many amazing people. However, this is only the start of my adventure and I have many questions yet to answer.

Dedication

For Sandy; your strength and belief helped me to achieve this.

'You can't stay in your corner of the forest waiting for others to come to you. You have to go to them sometimes'

Contents

List of Figures

List of Tables

Abbreviations

%	Percent
µl	Microlitre
(p)CRP	(porcine) C-reactive protein
ACTH	Adrenocorticotrophic hormone
AGP	Alpha-1-acid glycoprotein
ANOVA	Analysis of variance
AP-1	Activating protein 1
API	Acute phase index
ApoA-1	Apolipoprotein A-1
APP	Acute phase protein
APR	Acute phase response
BAC	Bacterial artificial chromosome
BES	Bacterial artificial chromosome end sequences
Bp	Basepair
BSA	Bovine serum albumin
Ca^{2-}	Calcium ion
cDNA	Complementary DNA
Cl^-	Chloride ion
CV	Co-efficient of variation
ddNTPs	Dideoxy nucleotides
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidephosphate
E. coli	*Escherichia coli*
EC	European Community
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FGF	Fibroblast growth factor
FPLC	Fast performance liquid chromatography
g	Gram
GLM	General linear model
GN	Genetic nuclear
Hr	Hour
h^2	Heritability
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
Hp	Haptoglobin
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IL	Interleukin
ITIH4	Inter-α-trypsin inhibitor heavy chain 4
Iαi	Inter-α-trypsin inhibitor

JAK	Janus kinase
KDa	Kilo Dalton
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
LW	Large White
M	Molar
MALDI	Matrix-assisted laser desorption/ionization
MAP	Mitogen activated protein
MAPK	Mitogen activated protein kinase
Mg_2Cl	Magnesium chloride
MHC	Major histocompatibility complex
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Na^{2+}	Sodium ion
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NF	Nuclear factor
NF-κB	Nuclear factor kappa B
nM	Nanomolar
NO	Nitric oxide
nt	Nucleotide
^{o}C	Degrees Celsius
O^{2-}	Superoxide ion
OD	Optical density
OH^-	Hydroxyl ion
OSM	Oncostatin-M
PBS	Phosphate buffered saline
PC/PCh	Phosphorylcholine/Phosphocholine
PC-BSA	Phosphorylcholic conjugated to bovine serum albumin
PCR	Polymerase chain reaction
PINI	Prognostic inflammatory and nutritional index
pmol	Picomol
PO_4^{2-}	Phosphate ion
PRRS	Porcine reproductive and respiratory disease
QL	Quantitative trail loci
r^2	Correlation coefficient
RBC	Red blood cells
RBP	Retinol-binding protein
REML	Restricted maximum likelihood
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay

RID	Radial immunodiffusion
RNA	Ribonucleic acid
RT	Room temperature
Sec	Second
S. aureus	*Staphylococcus aureus*
S. uberis	*Streptococcus uberis*
SAA	Serum amyloid A
SB-7	Chromogen reagent cocktail
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption/ionization
SEM	Standard error of the mean
SFR	Super fine resolution
SIV	Swine influenza virus
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
SRID	Single radial immunodiffusion
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TGF-β	Transforming growth factor beta
Th-1	T-helper 1 lymphocytes
Th-2	T-helper 2 lymphocytes
TIA	Turbidimetric immunoassay
Tm	Annealing temperature
TMB	Tetra methyl benzidine
TNF-α	Tumour necrosis factor alpha
TOF	Time-of-flight
TTR	Transthyretin
UTR	Untranslated region
UV	Ultraviolet
V_E	Environmental components of variance
V_G	Genetic components of variance
V_P	Total amount of variance in a phenotypic trait
w/v	Weight per volume

Base and SNP Codes

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
K	Guanine/Thymine
M	Adenine/Cytosine
R	Adenine/Guanine
S	Cytosine/Guanine
W	Adenine/Thymine
Y	Cytosine/Thymine

Chapter I

1. Introduction

As farming has become more intensive and consumers are more aware of animal welfare issues, animal health and welfare has become an area of increasing concern. The intensive pig production industry is especially aware of this with new regulations and quality assurance schemes in place. However, in order to meet these requirements, new reliable and rapid methods of monitoring health are required; the acute phase proteins appear to meet these requirements. These proteins are involved in the acute phase response of an animal, which is an early non-specific response of an animal to inflammation, trauma, infection or stress (Petersen *et al.*, 2004, Toussaint *et al.*, 1995).

1.1. Acute Phase Response

The acute phase response (APR) is an inflammatory response which occurs in animals as a consequence of tissue injury, trauma or infection (Eckersall, 1995, Baumann and Gauldie, 1994, Gruys *et al.*, 1994). It is part of the innate immune system and is a non-specific response in that it can be stimulated by a variety of factors including viral, bacterial, fungal or parasitic infection, physical trauma such as surgery, injuries, bone fracture or burns, childbirth and others. These conditions induce a localised inflammatory response releasing soluble mediators which in turn cause a systemic response as shown in Figure 1.1 (Ceron *et al.*, 2005, Gruys *et al.*, 1994). The systemic response is characterised by fever, anorexia and metabolic changes, changes in production of endocrine hormones and decreased levels of serum calcium, iron and zinc.

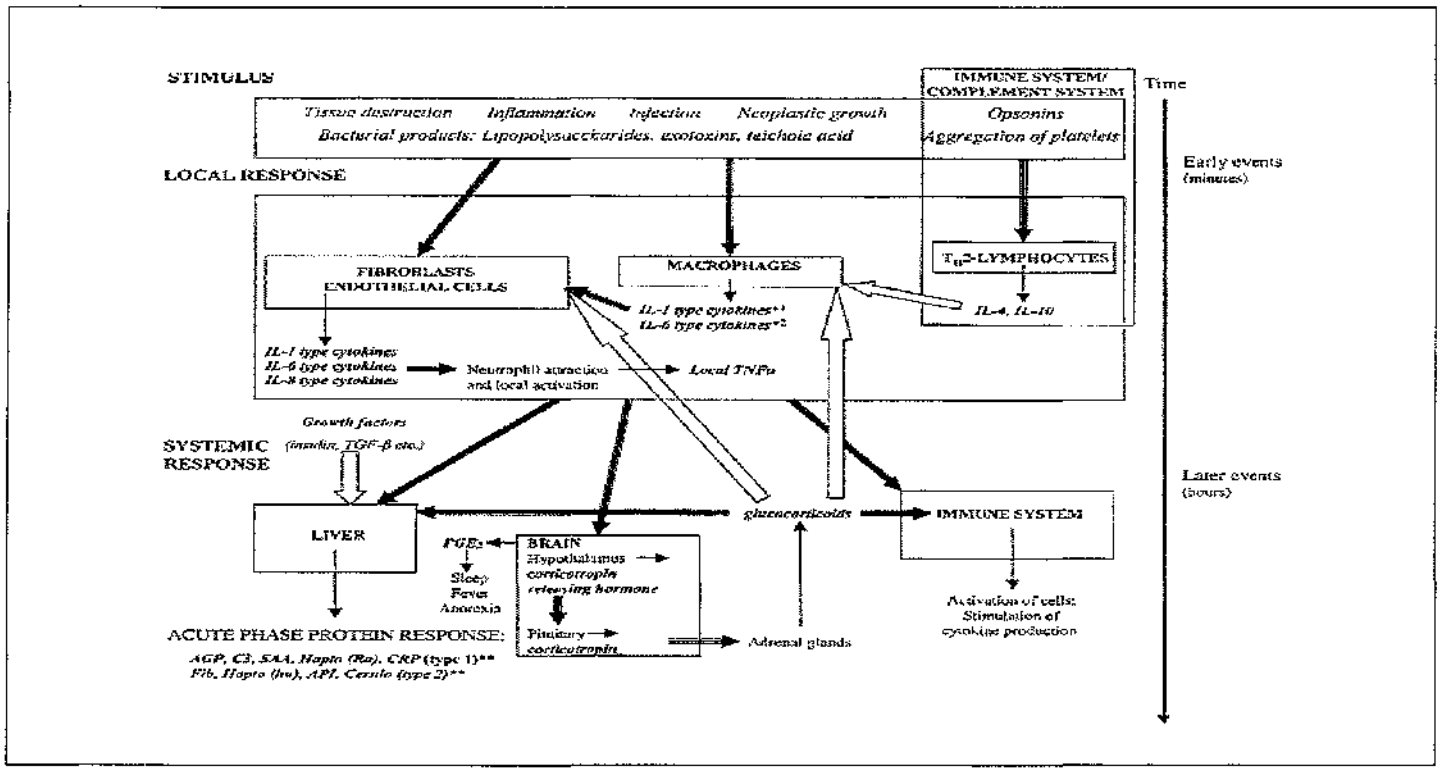


Figure 1.1: The acute phase response. Taken from (Petersen *et al.*, 2004). *Italics* indicate biologically active substances (hormones, cytokines etc.); ≡► : stimulating effect on cell type/organ given; ⇨: inhibiting effect on cell type/organ given; →: denotes a substance being secreted (italics) or an effect (non-italics) being caused by the given substance. *1: IL-1 type cytokines: IL-1α, IL-1β, TNF α, TNFβ in addition to the specific IL-1 inhibitor IL-1ra, the receptors IL-1RI, IL-1RII, TNF-RI and soluble receptors. *2: IL-6 type cytokines: IL-6, oncostatin M, ciliary neutrotrophic factor, IL-11, leukaemia inhibitory factor, all of which interact with receptors involving the common gp130 subunit. **Type1 acute phase proteins are predominantly induced by IL-1 type cytokines (synergistically with IL-6),while type 2 acute phase proteins are mainly induced by IL-6 type cytokines (and can be inhibitedby IL-1): AGP: α1-acid glycoprotein; C3: Complement factor C3; SAA: Serum amyloid A component; Hapto: Haptoglobin; CRP: C-reactive protein; Fib: Fibrinogen; API: α1-antitrypsin; Ceruloplasmin; Ra: Rat; hu: Human

Further details of the systemic response are given in Table 1.1 In addition, the APR causes changes in the concentrations of the plasma proteins also known as acute phase proteins (APP) (Ceron *et al.*, 2005, Trey and Kushner, 1995, Gruys *et al.*, 1994).

Table 1.1: Systemic changes of the acute phase response.

Overall Change	**Selected specific changes**
Neuroendocrine	Fever, anorexia, fatigue Increase in corticotropin-releasing hormone, cortisol and corticotrophin Increase in arginine vasopressin Decrease in insulin-like growth factor (IGF) I Increase in catecholamines
Hematopoietic changes	Leukocytosis Thrombocytosis
Metabolic Changes	Muscle catabolism Decrease in gluconeogenesis Increased lipogenesis Increase lipolysis
Hepatic changes	Increase in inducible nitric oxide synthase, heme oxygenase, metallothionein, tissue inhibitor of metalloproteinase-1
Other changes	Decrease in serum levels of calcium, zinc, iron, vitamin A and α-tocopherol Increase in serum levels of copper

(Gabay and Kushner, 1999, Trey and Kushner, 1995, Gruys *et al.*, 1994)

The purpose of these physiological changes is to aid in the restoration of homeostasis and to remove the cause of the disturbance. This may be effected by destroying any infective pathogens, removing harmful molecules or products and activating tissue repair processes (Moshage, 1997, Trey and Kushner, 1995). The APR can be viewed as a primitive response that aids in the survival of an animal following injury and as such, only lasts 24-48 hours before the animal returns to normal homeostasis or a specific

immune response occurs (Baumann and Gauldie, 1994). However, the APR can convert to a chronic response if the stimuli persist or the normal control mechanisms are disrupted. In these cases, tissue damage or further complications to the disease or injury may occur (Baumann and Gauldie, 1994).

1.1.1. Initiation of the Acute Phase Response

The APR is induced at the site of injury, specifically by tissue macrophages or blood monocytes. These cells are stimulated by mediators (e.g. transforming growth factor (TGF)-ß, toll-like receptors), released from cellular events such as mast-cell degranulation or platelet activation, by bacterial products such as lipopolysaccharide (LPS) and by the by-products of opsonins (Colditz, 2002, Suffredini *et al.*, 1999, Baumann and Gauldie, 1994). Once stimulated, the macrophages release a broad range of pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α. Cytokines are intercellular signalling polypeptides which have pleiotropic activity and can act both at a local and systemic level. The pro-inflammatory cytokines can be broadly divided into two groups; the IL-1 type cytokines including IL-1 and TNF-α, and the IL-6 type cytokines including IL-6, interleukin-11 (IL-11) and oncostatin M (OSM). (Gabay and Kushner, 1999, Baumann and Gauldie, 1994).

At a local level, IL-1 type cytokines activate the stromal cells including endothelial cells and fibroblasts resulting in a secondary wave of cytokines that initiates the cellular and cytokine cascades involved in the APR process (Petersen *et al.*, 2004, Gabay and Kushner, 1999, Baumann and Gauldie, 1994). Most cytokines are synergistic and are

often counter regulated by other cytokines and cytokine receptors. They may also act in an additive or co-operative manner with the effect that most cells are not exposed to a single cytokine, but rather to a complex mixture of cytokines and other inter- and intracellular messenger molecules (Fitzgerald *et al.*, 2001, Gabay and Kushner, 1999, Trey and Kushner, 1995).

At a systemic level, IL-1 and IL-6 type cytokines act upon various organs including muscle, brain, mammary gland and the liver. The liver is the principal target of the systemic cytokines where they activate or suppress the expression of the APP genes in the hepatocytes. In turn, this leads to production of the APP and causes changes in ion and metabolic pathways (Murata *et al.*, 2004, Yoo and Desiderio, 2003, Baumann and Gauldie, 1994). Studies in mice have shown that a single inflammatory stimulus can stimulate 5-10% of the protein encoding portion of the genome, a large proportion of which is involved with immunity or intracellular signalling (Yoo and Desiderio, 2003).

1.1.2. Regulation of the Hepatic Acute Phase Response

The regulation of the hepatic APR is under the influence of four major categories of factors; IL-1 type cytokines, IL-6 type cytokines, glucocorticoids and growth factors (i.e. fibroblast growth factor (FGF) and TGF-ß). The cytokines are able to induce APP production while the growth factors and glucocorticoids mainly act as modulators of cytokine action (Yeager *et al.*, 2004, Jensen and Whitehead, 1998, Moshage, 1997, Baumann and Gauldie, 1994).

1.1.3. IL-1 Type Regulation

There are two types of IL-1 receptor; type I (a transmembrane glycoprotein, ~80 kDa) and type II (a glycoprotein, ~60 kDa), both belonging to the IgG superfamily. The type I receptor is responsible for the transmission of the IL-1 signal. After ligand binding, the type I receptor is phosphorylated at serine/threonine residues, the ligand-receptor complex is then internalised and translocated to the nucleus, stimulating gene transcription. The type II receptor does not generate a signal (Fitzgerald *et al.*, 2001, Moshage, 1997).

There are also two types of TNF-α receptor; type I (55 kDa) and type II (75 kDa) both belonging to the IgG superfamily, however they are not homologous to IL-1 receptors (Fitzgerald *et al.*, 2001).

Once activated, both the TNF-α and IL-1 receptors initiate conversion of membrane sphingomyelin to ceramide via sphingomyelinase. The ceramide-activated protein kinases connect to several signalling pathways which lead to activation and translocation of the transcription factors activating protein 1 (AP-1) and nuclear factor (NF) κB which is involved in gene transcription of APP, many of which contain NFκB and AP-1 response elements in their promoter regions. NFκB is only activated after the phosphorylation and degradation of the inhibitory subunit IκB. The IL-1 signal also connects to the mitogen activated protein (MAP)-kinase pathway as does the signal of IL-6, connecting the two signalling pathways (Moshage, 1997, Baumann and Gauldie, 1994).

1.1.4. IL-6 Type Regulation

The IL-6 receptor (80 kDa) α subunit forms a complex with two signal-transducing gp130 ß-subunits, the gp130 subunit is common to all members of this family. Once activated, the IL-6 receptor complex activates JAK tyrosine kinases with subsequent tyrosine phosphorylation of the signal transducers and activators of transcription (STAT) proteins. Following phosphorylation, STAT protein homo- and heterodimerisation is induced and they are translocated to the nucleus where they bind to their response elements. This is through recognition of a CTGGGA motif in the APP genes (Moshage, 1997, Baumann and Gauldie, 1994).

1.1.5. The Role of Glucocorticoids and Growth Factors

Glucocorticoids and growth factors, such as TGF-β interact with the regulation of the APR in various ways but most important are their interaction with the cytokines (Gabay and Kushner, 1999). The glucocorticoids affect IL-1 type and IL-6 type cytokines in different ways. They have an inhibitory effect on IL-1 gene transcription and decrease the stability of IL-1 mRNA (Fantuzzi and Ghezzi, 1993). Glucocorticoids can also show an inhibitory effect on IL-6 type cytokines. However, more importantly, they cause a decrease in IL-6 receptors in monocytes but not in hepatocytes and are therefore considered essential for the induction of hepatocyte IL-6 receptors by both IL-1 and IL-6 (Fantuzzi and Ghezzi, 1993). Alongside the glucocorticoids, growth factors also interact with the APR. Transforming growth factor-β has been shown to increase IL-1 mRNA and modulate the effects of IL-6. In addition, TGF-β has been found to directly

affect APP production by decreasing production of albumin and apolipoprotein A1 mRNA in hepatocytes (Mackiewicz *et al.*, 1990).

1.1.6. Resolution of the Acute Phase Reaction

As continuation of the APR can cause tissue damage and complications, it is important that once it has achieved its function, the response can be rapidly ended. Despite the relatively short half-life of the cytokines involved, there are active inhibitory mechanisms in place, however the complete process is still unclear (Jensen and Whitehead, 1998, Baumann and Gauldie, 1994).

It does appear that the anti-inflammatory cytokines, IL-4 and IL-10, play significant roles in down-regulating the production of various cytokines. IL-4 is released by Th2 lymphocytes and causes the down-regulation of TNF, IL-1 and IL-8; it also enhances apoptosis of monocytes. IL-10 is produced by Th2 lymphocytes, monocytes, macrophages and B cells and inhibits synthesis of IL-1, TNF, IL-6 and IL-8. The actions of these and corticosteroid, which inhibits production of many initial and secondary cytokines, appear to regulate the termination of the APR (Jensen and Whitehead, 1998, Baumann and Gauldie, 1994).

1.2. Acute Phase Proteins

The acute phase proteins (APP) are defined as a group of plasma proteins that change in concentration by at least 25% due to either an internal or external trauma to an animal resulting in an APR. The change in concentration can be either an increase, termed

positive APP, (e.g. C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin (Hp)) or a decrease, termed negative APP (e.g. albumin, transthyretin (TTR)) (Koj, 1985, Pepys and Baltz, 1983, Kushner, 1982).

Once the APR is initiated, levels of serum APP change dramatically due to their altered production by the hepatocytes of the liver. The positive APP can be classified using the magnitude of their increase from baseline levels. They may be classified as major, for example CRP in man typically shows a 200 times increase; moderate, for example Hp shows a 10 times increase in pigs, or minor, such as ceruloplasmin which shows only a 50% increase from baseline levels in man (Eckersall, 2000b, Eckersall, 1995). The APP may also be divided into two groups dependent upon the cytokine group that induces them. Based on evidence from human, rat and porcine studies, Type 1 APP are induced by IL-1 type cytokines and synergistically by IL-6 type, while type 2 APP are induced by IL-6 type cytokines and are not stimulated by IL-1 type cytokines which may actually have an inhibitory effect on them (Gonzalez-Ramon *et al.*, 2000, Suffredini *et al.*, 1999, Heinrich *et al.*, 1990).

In humans and dogs, an APR will show a rapid reaction of 'major' APP within 4 hours of a trauma, these tend to be major type 1 APP and show a dramatic increase in concentration before rapidly returning to normal levels. The 'moderate' APP tend to be moderate type 2 APP which show a slower increase and may remain at elevated concentrations for up to two weeks before returning to baseline levels (Ceron *et al.*, 2005, Eckersall, 2000b). However it is important to remember that the APR is species specific in that each species shows a different response not only in the kind of APP produced but also the magnitude of their reaction and whether they are type 1 or type 2.

The one exception to this is that serum albumin appears to decrease in concentration by 10 – 30 % in all mammalian species (Petersen *et al.*, 2004).

1.3.Porcine Acute Phase Proteins

Due to its status as an intensively farmed animal and its use in biomedical research as models for human disease, transgenic research and organ transplantation, the APR of the pig has been of interest for many years. Since 1987, studies have taken place investigating the porcine APP and their responses to inflammation, disease and trauma, with the view to using them as markers of disease and animal welfare (Eckersall, 1987). Here, the various individual porcine APP will be described before their applications in veterinary science, meat production and animal welfare are discussed.

The porcine APP include many which are found in other species as well as one species specific APP; pig major acute phase protein (Pig-MAP) (although homologues are found in other species). The various porcine APP are listed in Table 1.2 in their categories of major, moderate, minor and negative. Of these: Hp, CRP, Pig-MAP and the negative APP, TTR, will be discussed in greater detail.

Table 1.2: Acute phase proteins of the pig

Major	Moderate	Minor	Negative
C-reactive protein	Pig-MAP	Fibrinogen	Albumin
Serum amyloid A		Ceruloplasmin	Transthyretin
Haptoglobin		α_1-acid glycoprotein	Apolipoprotein A-1
			Transferrin

(Parra et al., 2006, Itoh et al., 1993)

1.3.1. Haptoglobin

Porcine Hp is a plasma α_2 sialoglycoprotein that binds free haemoglobin (Hb). It is analogous to human haptoglobin type 1-1 and consists of two polypeptide chain types; α chains (~10 kDa) and heavier ß chains (50 kDa). The two subunits are held together by disulfide bonds which also crosslink the α subunits to form oligomers (Cigliano *et al.*, 2003, Heegaard *et al.*, 1998, Eurell *et al.*, 1990). Haemoglobin is an oxygen-binding tetramer ($\alpha 2ß2$) protein which contains a protoporphyrin ring complexed with heme. In humans and most likely pigs, the ß chain of Hb contains two specific binding sites for Hp while the Hb α chain contains one Hp binding site. The Hb $\alpha ß$ dimers bind stoichiometrically to Hp $\alpha ß$ subunits forming a soluble complex which enhances the toxic peroxidase activity of Hb (Petersen *et al.*, 2004, Langlois and Delanghe, 1996).

Haptoglobin performs a variety of roles in the affected animal but its primary function is presumed to be to prevent loss of iron and stop renal damage from occurring. When erythrocytes are destroyed, Hb is released into the circulatory system where it can pass through the glomerular filter and renal damage may occur. When Hp binds to Hb, the

complex is not filtered through the glomeruli but instead transported to the liver for protein degradation and Fe^{2+} recycling. This also has a bacteriostatic effect by restricting the Fe^{2+} availability required for bacterial growth (Cigliano *et al.*, 2003, Langlois and Delanghe, 1996, Eaton *et al.*, 1982).

In addition to the binding of free Hb, Hp also protects against free radical damage by superoxide (O^{2-}) and hydroxyl (OH) molecules. Free Hb allows the accumulation of OH molecules as Fe^{2+} can generate OH via the Fenton reaction; it can also catalyse the oxidation of low-density lipoproteins which damage vascular endothelial cells. The Hb-Hp binding complex thus prevents these occurrences (Cigliano *et al.*, 2003, Langlois and Delanghe, 1996). The Hb-Hp complex also prevents free Hb from interacting with endothelium-derived relaxing factor (EDRF) or nitric oxide (NO). Nitric oxide is produced by cells including cytokine-activated macrophages and large amounts are cytotoxic, providing a non-specific defence against microorganisms (Langlois and Delanghe, 1996).

Haptoglobin is also involved in immunomodulation via the binding of the Hb-Hp complex to CD11/CD18 receptors on granulocytes and monocytes. These receptors are involved in cell to cell and cell to matrix interactions. In addition, the Hb-Hp complex is also recognised via CD163, a specific surface receptor on macrophages, where it is then phagocytosed (Langlois and Delanghe, 1996). These roles and the other roles it performs are summarised in Table 1.3.

Table 1.3: Functions of selected acute phase proteins.

Acute Phase Protein	Function
Haptoglobin	Binds with haemoglobin Protects against free radical damage (anti oxidant) Bacteriostatic effect Immunomodulatory effect Angiogenic factor
C-reactive protein	Opsonisation and phagocytic removal of cell debris Activation of complement Modulation and interaction with platelets, macrophages, monocytes, lymphoid cells Binds with chromatin
Transthyretin	Transport of retinol and thyroxin

Porcine Hp has shown value as a diagnostic marker of a variety of conditions. In newborn piglets, baseline serum levels of Hp are initially very low, then reach a concentration of more than two-fold that of older animals before decreasing and returning to normal baseline levels of below 1 g/L at around 2-3 weeks of age (Martin *et al.*, 2005, Petersen *et al.*, 2004). There has been differences in baseline levels of Hp observed between sexes with boars having lower levels than sows, and also between herds, with conventional herds having higher levels than specific pathogen free (SPF) herds (Gymnich and Petersen, 2004, Petersen *et al.*, 2002b). During an APR, serum Hp concentrations generally increase more than 3-fold (but can be as much as 26-fold) and therefore it is considered a major porcine APP (Parra *et al.*, 2006, Heegaard *et al.*, 1998).

A rise in serum Hp can be induced by various means in pigs including inflammation caused by turpentine injection, infection with *Actinobacillus pleuropneumoniae,*

Mycoplasma hyorhinis, Streptococcus suis, or *Escherichia coli* and LPS intake (Sorensen *et al.*, 2006, Carroll *et al.*, 2004, Hulten *et al.*, 2003, Magnusson *et al.*, 1999, Dritz *et al.*, 1996, Eckersall *et al.*, 1996). Studies also showed that Hp concentrations can be used to follow the time course of an infection and as a marker of treatment efficacy with levels rising before clinical signs are apparent (Hulten *et al.*, 2003, Petersen *et al.*, 2002a).

In addition to infection, Hp can also be a marker of clinical signs as higher serum levels are associated with lameness, tail and ear bites, diarrhoea and respiratory disease. It can be associated with stress over a period of time (i.e. long-duration transport, routine changes) and can be used to differentiate between animals that will show a high and low weight gain at slaughter (Pineiro *et al.*, 2007b, Saco *et al.*, 2003, Petersen *et al.*, 2002a, Petersen *et al.*, 2002b, Eurell *et al.*, 1992).

The variety of studies carried out and the potential as a marker for disease, treatment efficacy, stress and weight gain means that porcine serum Hp levels could be an extremely valuable diagnostic tool.

1.3.2. C-Reactive Protein

C-reactive protein was the first APP to be discovered in humans in 1930 and has remained one of the most widely researched and measured of the APP (Tillett and Francis, 1930).

A member of the pentraxin family of proteins, CRP consists of five identical non-covalently associated subunits of 23.4 kDa each arranged symmetrically around a

naturally formed pore (Volanakis, 2001, Burger *et al.*, 1998). Based on the human structure, each subunit consists of 206 amino acids folded into two antiparallel ß sheets with a flattened jellyroll topology. Each subunit, all with the same orientation, has a recognition face with a phosphocholine (PCh) binding site consisting of two bound calcium ions (Ca^{2+}) and an adjoining hydrophobic pocket. Two oxygens of the phosphate group bind with the two Ca^{2+} while the choline group rests in the hydrophobic pocket. Two residues, Phe-66 and Glu-81 are also involved. Phe-66 provides a hydrophobic interaction with the choline methyl groups, while on the opposite end of the pocket, Glu-81 interacts with the positively charged choline nitrogen (Agrawal, 2005, Black *et al.*, 2004, Volanakis, 2001).

The opposite face of the pentamer is the effector face; it is on this side that the complement Clq-binding site is located and the immunoglobulin FcR binding site is presumed to be. Residues including Asp-112 and Tyr-175, situated along a cleft extending from the centre of the sub-unit to the central pore are responsible for this binding, leading to complement activation. Compared to PCh binding, calcium is not required for Clq binding, however Clq must bind to more than one CRP pentamer before complement activation can occur (Agrawal, 2005, Black *et al.*, 2004, Volanakis, 2001). This ability to bind not only PCh but also to initiate the classical complement system links the non-specific immune response to the specific adaptive immune response (Murata *et al.*, 2004).

C-reactive protein plays a variety of important roles in protection against infection and restoration of healthy tissue by eliminating damaged cells via the complement system and phagocytic cells. The primary CRP ligand, PCh, is found in the teichoic acids,

capsular carbohydrates and lipopolysaccharides of bacteria and other organisms. It is also present in the outer leaflet of most biological membranes as the polar head group of lecithin and sphingomyelin (Murata *et al.*, 2004, Volanakis, 2001). However, in normal cells these polar heads are inaccessible to CRP; therefore CRP can only bind to these molecules in damaged or apoptotic cells.

Once CRP is bound to the surface of a damaged cell, it initiates the formation of C3 convertase. This leads to binding of opsonic fragments of C3 to the activating surface of CRP. Phagocytosis of the opsonised particles and apoptotic cells can then take place through binding to the IgG receptor, FcR, on monocytes and macrophages (Black *et al.*, 2004, Volanakis, 2001). The role of CRP in phagocytosis and other roles it plays are summarised in Table 1.3.

Porcine CRP was first detected in plasma samples taken from piglets with pyrexia (Eckersall, 1987). Later studies have looked at baseline and acute phase concentrations of CRP under various conditions and induced by different means including turpentine oil injection, experimental inoculation and stress (Carroll *et al.*, 2004, Eckersall *et al.*, 1996). The baseline concentration of serum CRP in adult pigs is generally accepted to be under 100 mg/L and during an APR increases 10 – 15 times, categorising it as a major porcine APP (Parra *et al.*, 2006, Lampreave *et al.*, 1994). CRP often reacts faster and returns to normal quicker than most of the other porcine APP which makes it an ideal candidate as an early indicator of infection (Parra *et al.*, 2006, Lauritzen *et al.*, 2003, Heegaard *et al.*, 1998). At the present time, CRP concentrations have been studied in various disease models and overall levels correlate to the time course of treatment and severity of infection. Similar patterns have been observed in animals

under stress due to transport or changes in routine (Pineiro *et al.*, 2007b, Pineiro *et al.*, 2007c). It has suggested that CRP can be used to identify animals with sub-clinical disease and in the assessment of stress (Murata *et al.*, 2004, Heegaard *et al.*, 1998, Eckersall *et al.*, 1996).

1.3.3. Pig-Major Acute-Phase Protein

Pig-Major Acute-phase Protein (Pig-MAP) is a porcine specific plasma α2-globulin (115 kDa) which is homologous to inter-α-trypsin inhibitor heavy chain 4 (ITIH4) in humans. Both are members of the inter-α-trypsin inhibitor (IαI) heavy chain family (Martin *et al.*, 2005, Pineiro *et al.*, 1999, Lampreave *et al.*, 1994).

The IαI family is a group of related plasma protease inhibitors. These proteins are formed from a variety of multi-polypeptide molecules, each with different assemblies from a group of four distinct polypeptides. This group consists of three related heavy (H) chains; H1, H2, H3 and a light chain called bikunin. The bikunin chain contains two Kunitz-type protease inhibitor domains which are bound to the heavy chains via glycosaminoglycan bridges (Pineiro *et al.*, 1999, Salier *et al.*, 1996). However, although Pig-MAP (and ITIH4) shows significant sequence homology with H1, H2 and H3, they do not link to bikunin and subsequently they lack the protease inhibitory function. It is not clear what function Pig-MAP performs during an APR but it may play a role during gestation by protecting the uterus from the inflammatory response induced by the attachment of the conceptus to the uterine epithelium (Martin *et al.*, 2005).

Martin *et al.* (2005) showed that during the first days of life, Pig-MAP serum concentrations increased rapidly from close to 0 to 1.6 g/L and remained high until weaning before dropping to adult baseline concentrations of around 0.4 0.6 g/L (Martin *et al.*, 2005, Carpintero *et al.*, 2005). In an APR, Pig-MAP serum concentrations rise by at least five times and remain elevated for several days with a maximum concentration reached 24-48 hours after initiation of the APR (Heegaard *et al.*, 1998, Alava *et al.*, 1997).

A rise in serum Pig-MAP can be induced by turpentine injection, *A. pleuropneumoniae*, *S. suis*, Aujeszky's virus and surgery. In addition, pigs exposed to stressful situations consistently show a rise in Pig-MAP levels (Pineiro *et al.*, 2007c, Heegaard *et al.*, 1998, Alava *et al.*, 1997).

As a pig specific APP, Pig-MAP shows great promise as a marker of infection and serum levels show a good correlation with disease. However, more extensive and wider-ranging studies need to be performed to examine its function and concentrations under differing conditions.

1.3.4. Alpha-1-Acid Glycoprotein

Alpha-1-Acid glycoprotein (AGP), also known as orosomucoid, is a negatively charged glycoprotein of 41-43 kDa, of which 45% consists of carbohydrate. It is considered part of the immunocalin family, a family of proteins which modulate immune and inflammation. The precise function of AGP is unknown but studies have shown that it

may depend on the carbohydrate content which alters during an immune event (Hochepied *et al.*, 2003, Fournier *et al.*, 2000).

At birth, AGP concentrations in piglets are extremely high (14.3 mg/ml) and gradually decrease to adult baseline concentrations (1.4 mg/ml) by 20 weeks of age (Itoh *et al.*, 1992). However, there are conflicting opinions as to whether AGP should be considered an APP in pigs. This stems from the lack of response of AGP to LPS stimulation suggesting that AGP may only be a minor indicator of inflammation (Eckersall *et al.*, 1996, Lampreave *et al.*, 1994). Despite the lack of response to LPS, AGP does show significantly higher concentrations in pigs exposed to *A. pleuropneumoniae* and *M. hyopneumoniae* indicating that it may be useful as a potential marker of disease, however more research is needed in this area (Itoh *et al.*, 1992).

1.3.5. Negative Acute Phase Proteins

The concentration of negative APP decreases during the APR, this may allow the amino acids used in their production to be diverted to the production of APP which will aid with immediate survival of trauma or infection, however the exact function is unknown. In pigs, the negative APP include albumin, transferrin, transthyretin (TTR) and apolipoprotein A-1 (ApoA-1) with the latter two being particularly indicative of an APR (Campbell *et al.*, 2005, Lampreave *et al.*, 1994).

1.3.5.1. Transthyretin

Transthyretin is a homotetrameric protein (55 kDa) consisting of 4 identical subunits each 127 amino acids long. Each monomer is made up of 8 β-strands organised into 2 four stranded β-sheets and 1 short α-helix. The 2 dimers create a channel where 2 hormone binding sites are located and two retinol-binding protein (RBP) binding sites are found on the surface of the molecule (Eneqvist *et al.*, 2003, Monaco *et al.*, 1995). The porcine isoform of the protein shares 85% homology with the human form, and differs by the addition of 3 extra amino acids at the C-terminus (Gly-Ala-Leu), this gives it a subunit length of 130 amino acids (Duan *et al.*, 1995).

Transthyretin is a transport protein, and as such, is involved in the transport of thyroxine and RBP in complex with vitamin A. Without the presence of transport proteins such as TTR, thyroxine would move into the lipid membranes from the blood, causing an uneven distribution of the hormone (Eneqvist *et al.*, 2003, Duan *et al.*, 1995). In humans, decreasing serum levels of TTR are used as indicators of malnutrition and have a strong correlation with patient outcome; continually low serum concentrations indicate a requirement for nutritional support (Beck and Rosenthal, 2002).

Recent studies in pigs have shown baseline levels to be ~ 300μg/ml with the concentration falling significantly following infection with *S. suis* (Campbell *et al.*, 2005). However, unlike CRP, Hp and Pig-MAP, there appears to be no significant effect of stress on TTR levels (Pineiro *et al.*, 2007b, Pineiro *et al.*, 2007c)

As with other APP, more studies need to be carried out to define not only the precise functions of this APP but also differences in concentrations due to age, breed, farm location and health status and also their values of markers of infection.

1.4. Applications of Acute Phase Proteins in Veterinary Diagnostics

As has been discussed, porcine APP can be used to not only monitor inflammation but also as markers of disease and sub-clinical infection. These properties make them a useful tool in porcine health and pig production systems.

1.4.1. Marker of Health Status

Many studies have focussed on measuring APP during infection with various infectious agents; in particular Hp is recognised as an especially valuable non-specific marker of disease. On farms, raised APP levels can be used to identify animals with sub-clinical disease. This allows not only early treatment of infection but would allow isolation of an infected animal, hence preventing the spread of disease within intensive systems (Petersen *et al.*, 2004, Knura-Deszczka *et al.*, 2002, Eckersall, 2000a). Animals suffering from disease also suffer from associated starvation and therefore negative energy balance as muscles are catabolised to provide amino acids for APP production and as an energy source (Gruys *et al.*, 2005). Early detection of an APR would ensure treatment of these animals and allow feed formulations to be adapted to a change in nutrient requirements such as increased tryptophan or L-arginine (Bruins *et al.*, 2002). This would ensure that animals do not lose condition and hence help to avoid lower profit margins.

1.4.2. Marker of Health and Welfare

It has been accepted, for some time now, that stress in production animals can have detrimental effects on their performance. Pigs are known to be susceptible to stress, particularly those carrying the halothane gene. Stress in the animal can be associated with a variety of factors including environment, handling, transport, social environment, genetics and the sex of the animal (Salak-Johnson and McGlone, 2007, Moberg and Mench, 2000). These studies have shown that Hp, Pig-MAP and CRP can be used as indicators of stress due to transport, routine disruption and environmental factors such as hygiene, with levels of these APP rising in response to the perceived stress. However, it can be difficult to separate the effects of trauma or subclinical disease from the effects of stress (Pineiro *et al.*, 2007b, Saco *et al.*, 2003). It is biologically possible for stress to cause a change in APP concentrations through the sympatho-adrenal and hypothalamic-pituitary-adrenal (HPA) axes. Activation of these axes, results in production of catecholamines and glucocorticoids, both of which are involved with cytokine induction and therefore APP production (Murata, 2007).

The measurement of APP could have potential use in not only identifying animals suffering from severe stress but also in general welfare management, i.e. high APP levels indicate underlying problems whether these are hygiene or disease related (Murata, 2007). This would not only be of use to the stockman but could also be incorporated into the welfare schemes now associated with pork production.

1.4.3. Use in Meat Inspection

As meat production has become increasingly industrialised and food safety has become of paramount importance to both producer and consumer, additional and more efficient methods of assessing meat quality have been sought. Many investigations into this area have centred on blood based sampling of animals entering the production line and the use of on-farm tests in the form of biosensors (Hiss *et al.*, 2003, Toussaint *et al.*, 1995). As APP levels have been shown to be consistently elevated in those animals with sub-clinical infection, inflammation and stress (all with adverse effects on meat quality or hygiene), they could provide an ideal screening tool in meat production systems (Eckersall, 2000a, Saini *et al.*, 1998, Toussaint *et al.*, 1995). Studies have shown that APP concentrations can be measured in meat juice and that the measurements correlate with Hp serum levels (Gutierrez et al., 2008, Hiss et al., 2003). Currently, meat juice can be used as an assessment of pork quality therefore it may be possible to incorporate the measurement of APP into post-slaughter tests at the abattoir. This would provide not only an indication of pork quality but also general health and welfare status (BPEX, 2002).

1.5. Acute Phase Index

The use of APP in veterinary diagnostics has major potential but there are several APP to choose from and some may be better indicators or markers than others for particular diseases. It is possible that this can be overcome with the use of an acute phase index (API) based upon the prognostic inflammatory and nutritional index (PINI) used in human patients and the bovine API (Gruys *et al.*, 2006, Ingenbleek and Carpentier, 1985). This index uses a selection of values from fast and slow reacting positive and

negative APP to give a more reliable parameter for measuring health than the individual APP values and a preliminary formula has been suggested for use in pigs:

$$\frac{\text{CRP (or SAA) x Hp (or plasma viscosity)}}{\text{Albumin x Vit. A*}}$$

* TTR could be substituted for Vit. A as during an APR they are found in complex with one another.

However, further studies are required to verify this and calculate acceptable values before it can be used (Gruys *et al.*, 2006, Toussaint *et al.*, 1995).

1.6. Genetics of Acute Phase Proteins

In order to be useful as markers of disease, it is necessary to understand the relationship between the production of the APP in question (both at baseline and stimulated levels) and disease. By looking at the genetics of the APP, we can gain an understanding of what regulates their production and identify any associations with disease and/or the organism's 'normal' status. However, very few studies have been carried out in livestock species including pigs. In recent years, a great deal of research has centred around the genetics of the human APP, in particular CRP, and at the present time, this is the most understood of the APP, although some studies have been and are being carried out into other APP such as haptoglobin, transthyretin and ITIH4.

1.6.1. Genetic Studies of Human Acute Phase Proteins

1.6.1.1. Haptoglobin

Haptoglobin in humans is located on chromosome 16q22 and has a polymorphism in the α protein giving two variants of HP-1. This has led to three major phenotypes: HP-1,1, HP- 2,1 and HP-2,2 (NB: porcine Hp is homologous to HP-1,1). Studies have shown that Hp serum levels vary with Hp phenotype, typically in the order HP-1,1 > HP-2,1 > HP-2,2. (Cox *et al.*, 2007, Langlois and Delanghe, 1996). A single nucleotide polymorphism (SNP) located in the promoter region of *Hp* is thought to be responsible for the differences in phenotype. Single nucleotide polymorphisms occur when one nucleotide is substituted for another. They can be located in the coding regions of genes and if they result in an amino acid change they can directly affect protein function (Beuzen *et al.*, 2000). The SNP, a 61A–C substitution, is associated with decreased Hp levels with the C-allele closely associated with undetectable Hp levels (Maeda, 1991). In addition, a novel SNP 101C–G substitution has also been found to be associated with serum Hp concentrations (Teye *et al.*, 2003).

Presently, no heritability studies have been carried out for Hp in human populations, however, studies indicate that Hp genotype is related to race. The two SNP mentioned above are found in persons of African origin whilst other SNP are found in persons of Asian and European descent (Teye *et al.*, 2006). This means that if Hp genotype is to be used as a biomarker, then race must be taken into account during testing and when performing in-depth studies.

1.6.1.2. C-Reactive Protein

The *CRP* gene is located on human chromosome 1q23 (Walsh *et al.*, 1996), and consists of two exons and one intron containing a polymorphic (GT) repeat (Szalai *et al.*, 2002, Lei *et al.*, 1985). Thirteen alleles of the GT repeat were identified, corresponding to introns containing 9 to 25 repeats (GT^{9} to GT^{25}) and giving rise to 38 genotypes. Three of these genotypes $GT^{16/16}$, $GT^{16/21}$ and $GT^{21/21}$ account for 78% of all Caucasians. The GT^{16} and GT^{21} alleles were designated CRP^{low} alleles and further analysis revealed that the number of CRP^{low} alleles carried by an individual significantly affected CRP levels. Specifically, those individuals carrying the CRP^{low} alleles had a two-fold lower CRP serum concentration than those individuals carrying other genotypes (Szalai *et al.*, 2002).

Further studies have examined the association between SNP and serum concentrations. A number of SNP have been detected in *CRP* that have associations with CRP concentrations (Wang *et al.*, 2006, Suk *et al.*, 2005, Brull *et al.*, 2003). Some of the SNP published are found to be in linkage disequilibrium (LD) with each other which may explain some of the associations, however, those SNP that are not in LD may have individual effects on CRP levels or may work in tandem (Miller *et al.*, 2005). More work is needed in this area, but it is clear that CRP levels are under genetic control.

Large scale familial studies have also been carried out to obtain an estimate of CRP baseline concentration heritability in order to determine what proportion of the total variation can be attributed to genetic effects. The studies found that CRP levels have a high heritability (h^2) with estimates of 0.39, 0.4 and 0.52 (MacGregor *et al.*, 2004, Vickers *et al.*, 2002, Pankow *et al.*, 2001).

1.6.1.3. Interleukin-6

It should be noted, that studies have also reported that polymorphisms within the IL-6 gene are significantly associated with CRP levels (Vickers *et al.*, 2002). As previously discussed, *IL-6* is involved with the expression of CRP and as such it is hypothesised that a SNP in *IL-6* could affect CRP levels via IL-6 gene transcription and the subsequent effect of differing cytokine levels on CRP gene expression (Vickers *et al.*, 2002). However, further studies will be needed to clarify this hypothesis.

1.6.1.4. ITIH4 and Transthyretin

Both ITIH4 (human homologue to Pig-MAP) and TTR have been associated with pathological conditions in a limited number of studies. The *ITIH4* gene is located on human chromosomal region 3p21.1-p22 which has been described as a possible quantitative trait locus (QTL) for the condition dyslipidemias (Yuan *et al.*, 2000, Jean *et al.*, 1997). Single nucleotide polymorphisms have been located in this gene and in particular a C/T substitution within intron 17. Although no association studies have been carried out between this SNP and ITIH4 concentrations, an association has been found between the polymorphism and total plasma cholesterol concentration. Specifically it was found that the C allele is associated with significantly higher cholesterol concentrations (Fujita *et al.*, 2004).

The *TTR* gene is located on human chromosome 18q12.1 and is composed of four exons (Tsuzuki *et al.*, 1985). Many SNP (>80) within the *TTR* gene have been identified, a number of these are associated with amyloidosis and cardiomyopathy and cause a mutated version of the protein to be synthesised (Saraiva, 2002). The *TTR* gene has also

been reported to be implicated with the pathophysiology of schizophrenia. However an association study between genotype and TTR levels carried out as part of a wider study looking at schizophrenia found that there was no association between serum levels and the genotypes of the two SNP studied in this case (Ruano *et al.*, 2007).

Using the evidence gained from genetic studies of the human APP, it can be predicted that some, if not all of the porcine APP may be under the control of polymorphisms and/or may be associated with certain pathological conditions or immune function. Studies have mapped the porcine APP genes and these will be discussed in detail in Chapter 4. If porcine APP genes are shown to be under genetic control, this may have implications for commercial breeding companies, particularly in terms of breeding for immune function or disease resistance.

1.7.Breeding for Disease Resistance

Disease can be a major issue in intensive breeding systems causing animal welfare problems and ultimately leading to a loss of profit. At the present time, disease control can be broadly divided into three main areas;

- Preventative: vaccination, controlled entry into animal housing
- Treatment: antibiotics, antihelminthics, culling when necessary
- Breeding for disease resistance

The first two have been the methods of choice for many years, however they do have disadvantages, namely cost, consumer demand for produce free from antibiotics,

availability of drugs, legislation and ensuring that treatment and prevention programs are followed (Visscher *et al.*, 2002, Muller and Brem, 1991). The third option, breeding for resistance, is becoming more viable and is possibly a favourable alternative. The advantages of this method include simplicity and low cost (when a breeding program is in place), improved animal health and welfare and increasing availability of molecular markers as selection tools (Nicholas, 2005, Montaldo and Meza-Herrera, 1998). It is not without disadvantages, although there are compelling arguments against them. Stear *et al.* (2001) divided the disadvantages into three areas; sustainability, feasibility and desirability and argued that these concerns could be addressed.

Genetic resistance to specific diseases has been recognised for some time, examples include *Brucella suis* and *E. coli* in pig, foot rot in sheep and scrapie in sheep (Chang and Stear, 2006, Drogemuller et al., 2001, Whittington and Nicholls, 1995, Cameron et al., 1942). In these instances, the genotype susceptible to the disease has been identified. The use of molecular markers has allowed individuals carrying the susceptible genotype to be either culled or withdrawn from the breeding population (Wilkie and Mallard, 2000).

Breeding for generalised resistance to disease and in particular sub-clinical disease is more problematic as the desired phenotypic traits have first to be identified. The innate immune system appears to be a logical target as it is non-specific and can recognise a large number of pathogens, as previously discussed. In addition, various components of the innate immune system can be easily measured and associations with disease susceptibility and production traits can be calculated (Clapperton *et al.*, 2005b, Colditz, 2002).

1.7.1. Breeding for Resistance in Pigs

In pigs, a number of studies have centred on aspects of innate immune traits and have identified several areas of interest. One of the most important, from a breeding perspective, is that pigs show genetic variation both within and between breeds in innate immune traits such as monocyte number, interleukin-2, neutrophil number and porcine mannan-binding lectin A which has also been shown to be heritable (Landrace h^2=0.8, Duroc h^2=0.15) (Juul-Madsen et al., 2006, Clapperton et al., 2005b). These results indicate that this may lead to variation in disease resistance which could be a useful breeding tool. It is also possible that the response of an animal to disease may be of an interest, if an immune trait can be measured before and after an immune event, it would be possible to determine whether the response of an animal is determined by its genetic background or whether this is a heritable trait.

The other main area of interest is associations between innate immune traits and production traits. Studies by Clapperton et al (2005) and Galina-Pantoja et al (2006) reported that immune traits can have strong associations with production traits in healthy animals and that these associations can have negative effects. In particular, lymphocytes expressing CD16+, CD2+/CD16+ and CD8+ receptors were associated with reduced average daily gain, with a higher frequency of these lymphocytes having an overall negative effect on production traits (Galina-Pantoja *et al.*, 2006). Interestingly, a similar study found no association with CD8+ and performance but did find that as monocytes, NK cells and B cell proportions increased the performance of the pigs decreased (Clapperton *et al.*, 2005a). In contrast, earlier studies used the production of Yorkshire pigs bred for HIR (high) and LIR (low) antibody and cell-mediated immune response. In these studies, HIR pigs had a better rate of weight gain

and responded better to vaccination, however some stimuli provoked more inflammation than in LIR pigs (Wilkie and Mallard, 1999).

Although it appears to be possible to breed for innate immune traits, there is an effect on production traits and this needs to be taken into account before breeding schemes are put in place.

As has been discussed, the use of porcine APP as biomarkers of health is a possibility for future pig production but more research is required to increase the basic understanding of the porcine APP concentrations and the factors influencing them. The objectives of the work presented in this thesis are;

a) to determine the baseline concentrations of 4 porcine APP (Hp, CRP, Pig-MAP and TTR) in a high health boar population and identify any differences between breeding lines;
b) to identify polymorphisms in the 4 porcine APP genes and use them to genotype a population of high health boars;
c) using the data gained from objectives a) and b), to determine whether there is an association between APP genotypes and APP baseline concentrations;
d) to carry out a study of the heritability of APP baseline concentrations in high health pigs.

Chapter II

2. Development of an Assay for the Measurement of C-Reactive Protein in Porcine Serum

2.1. Introduction

The measurement of APP in pigs provides a non-specific indication of infection, inflammation or trauma. C-reactive protein is considered a major APP in pigs and is therefore particularly useful as a marker of disease. Although commercial assays were available to measure porcine CRP in serum; with a high number of samples to be processed these can prove to be costly. Therefore the development of an in-house assay for porcine CRP (pCRP) was undertaken with the objective of establishing a reliable, robust and more cost effective method than those previously available.

2.1.1. Use of Immunoassays in Measuring Acute Phase Proteins

The majority of assays used for measurement of APP in livestock are now reliant upon the use of antibody-based assays. The main exception to this is the automated biochemical Hp assay which is based on haemoglobin (Hb) binding (Eckersall *et al.*, 1999); this will be discussed in further detail in Chapter 3.

2.1.2. Background

The immunoassay first originated in 1959 with an assay for insulin and is based upon the reversible reaction between an antigen and its specific antibody (Yalow and Berson, 1959). Since then, immunoassays have been developed to include a wide range of techniques for the detection and quantification of the antibody-antigen reaction, and an enormous selection of different labels and detection systems are now available; for example, fluorescence and chemiluminescent systems (Ronald and Stimson, 1998).

The first immunoassays were based upon the use of radioisotope labelling, known as radioimmunoassay (RIA). The antigen of interest was labelled with a radioisotope which competed with the unlabelled antigen (present in the sample) for binding to the antibody. After incubation, the bound and free fractions were separated and the radioactivity of the separated fractions measured (Wu, 2006). However, this technique had its disadvantages, in particular the health hazards and waste disposal problems associated with working with radioactive isotopes.

The original RIA used polyclonal antibodies from human sources or those produced in animals. The problems of limited supply and multispecific binding were overcome by the discovery and production of monoclonal antibodies (Kohler and Milstein, 1975). These antibodies can have a high specificity towards the original antigen and large quantities of monoclonal antibodies with the same specificity can be produced. Both polyclonal and monoclonal antibodies are commonly used in immunoassays with the choice of antibody being dependent on antibody specificity and assay performance optimisation (Wu, 2006, Ronald and Stimson, 1998). One of the advantages of antibody-based methods is the wide range of assay formats available including single

radial immunodiffusion (SRID), enzyme linked imunosorbent assay (ELISA) and immunoturbidimetric assays (Eckersall, 1995). The most common of these assays will be discussed in relation to measuring APP, and more specifically porcine CRP.

2.1.2.1. Single Radial Immunodiffusion

Single radial immunodiffusion (SRID) is an immunoprecipitation technique in which the antigen is placed in a well within an agar gel containing antibody and diffuses out into the gel. A ring-shaped precipitate forms when antibody and antigen are present in the correct proportions so that the ring diameter correlates with the concentration of antigen. The diameter of the rings is measured and the test can be calibrated using standards of known concentration (Mancini *et al.*, 1965). The SRID is a simple and low cost method of determining antigen concentration. However it has disadvantages such as the incubation time (24 – 48 hrs), the sensitivity of the technique and the possibility of human error in measuring the precipitate ring. Porcine CRP has been measured using SRID and has a high correlation with some ELISA results, however, it is not the method of choice due to the incubation time and sensitivity, particularly with low concentrations (Burger *et al.*, 1998).

2.1.2.2. Sandwich or double antibody ELISA

The double antibody (sandwich) is the most common format for this type of immunoassay due to its high specificity, and uses two antibodies, ideally raised to two different epitopes of the antigen. Using two specific antibodies improves the specificity of the assay as generation of the signal requires both antibodies to bind. The capture

antibody is linked to a solid support such as the walls of ELISA plate wells and the sample dispensed in buffer into the well (this may be preceded by a blocking step). Any antigen in the sample will bind to the antibody so that when a second, generally enzyme labelled (for example, alkaline phosphatase) antibody is applied, the concentration of antigen can be detected by use of the signal generated from the enzyme labelled antibody. This format has the advantages of being highly specific and sensitive, although it cannot be used for small molecules or where two separate binding sites are not available (Ronald and Stimson, 1998). Commercial sandwich ELISA are available and have been validated and used in studies of pig APP (Tecles *et al.*, 2007, Parra *et al.*, 2006, Heegaard *et al.*, 1998).

2.1.2.3. Ligand-Binding ELISA

This assay uses a ligand linked to a solid support in order to capture the antigen. An antibody can then be applied, followed by a secondary enzyme conjugated antibody to detect the antigen. This approach has been used to measure both canine and porcine CRP. In both cases, the ligand is phosphorylcholine conjugated to bovine serum albumin (PC-BSA). The phosphorylcholine binds to CRP in the presence of $CaCl_2$ (Sorensen et al., 2006, Eckersall et al., 1996, Eckersall et al., 1989b). In the porcine assays, Sorensen *et al.* (2006) used an anti-pig CRP monoclonal antibody whilst Eckersall *et al.* (1996) used an anti-human CRP antibody which showed cross-reactivity to porcine CRP. The cross reactivity of anti-human CRP to porcine CRP has been validated for use in ELISA, and this has been exploited for use in other assay formats (Kjelgaard-Hansen *et al.*, 2007, Lauritzen *et al.*, 2003, Burger *et al.*, 1998).

2.1.2.4. Turbidimetric Immunoassays

Turbidimetric immunoassays (TIA) involve the detection of an antibody-antigen complex either by eye (formation of a visible precipitate) or by quantitative methods e.g. turbidity or nephelometry, based on the change in light absorption or change in light scattering properties of the complex. Antibody readings can be enhanced by the use of particles when the antibody or ligand is adsorbed onto the particle, which causes a larger or more easily detected complex to be formed in the presence of antigen and allows lower concentrations to be measured more effectively (Ledue and Rifai, 2001, Ronald and Stimson, 1998). The relationship between antigen and antibody is shown in Figure 2.1 and it is important that the optimum antibody to antigen ratio (below the area of equivalence) is used to avoid false and highly inaccurate results. Advantages of TIA are the ability to use automated analysers giving high throughput of samples, and in some cases they have extremely high sensitivity (Ledue *et al.*, 1989). Using the cross reactivity reported between anti-human CRP and porcine CRP, two commercial human TIA have been validated for use with porcine samples. The use of these two assays shows the potential of using cross-reactivity between species, however, each batch of antibody must be validated before use (Kjelgaard-Hansen *et al.*, 2007).

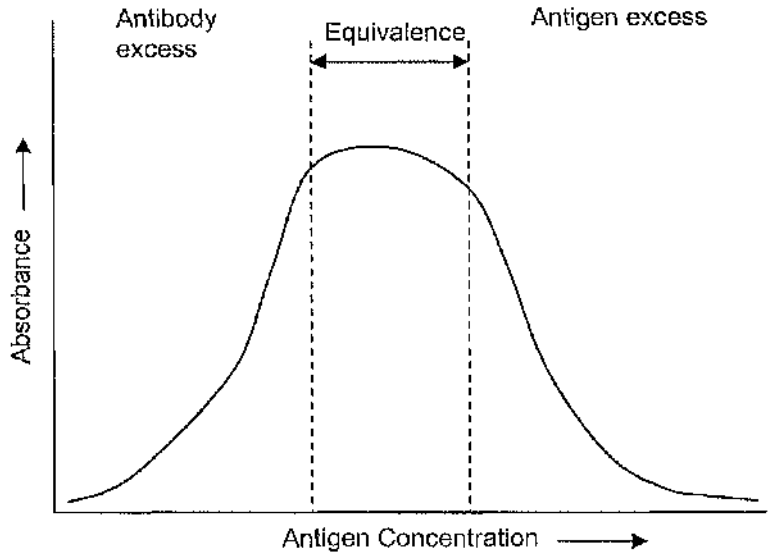


Figure 2.1: The Heidelberger-Kendall curve showing the general relationship between antigen concentration and absorbance (turbidity).

The aim of this study was to develop and validate a sensitive immunoassay to allow quantitative measurement of CRP in porcine serum. Initially samples were measured using a commercial ELISA assay; however development of an 'in-house' assay was considered more desirable. Several approaches were tried in the development of the in-house assay for pCRP including TIA and ELISA (both sandwich and ligand-binding). After assessing the alternatives, a ligand-binding ELISA using PC-BSA and anti-human CRP was chosen as the most suitable method and validated using the commercial assay as a comparison method. This ELISA was then used to determine CRP concentrations in a population of high health boars (See Chapter 3) and their progeny (See Chapter 5).

2.2.Material and Methods

All reagents were obtained from Sigma-Aldrich, Poole, UK unless otherwise stated. Double distilled water was used throughout.

2.2.1. Samples

A selection of porcine serum samples were made available for use in assay validation from the Faculty of Veterinary Medicine, University of Glasgow. The CRP content of these samples had been previously measured using the Tridelta PHASE™ RANGE Porcine C-reactive Protein Assay (Tridelta Development Plc., Co. Wicklow, Ireland) with samples being stored at -20°C until required. Additional serum samples were prepared from porcine blood collected from local abattoirs. This was incubated at 37°C for 30 min before the unclotted fraction was removed and centrifuged at 1853 xg for 10 min to remove debris. The resulting serum was stored at -20°C until required.

2.2.2. Determination of CRP

The CRP content of additional serum samples, the standard pool and the internal controls for the ELISA was quantified using the Tridelta ELISA. This is a commercially available kit and has been extensively used in previous published studies, as such, it was considered validated for use as a reference tool. The ELISA is a solid phase sandwich immunoassay using a horse-radish peroxidase (HRP) conjugated anti-porcine CRP antibody.

The assay was used according to the manufacturer's protocol. Briefly, samples were applied at a 1:100 dilution in assay buffer to precoated microtitre plates and incubated for 15 min. After washing, the monoclonal conjugated antibody was applied and incubated before washing again. Tetra methyl benzidine (TMB) substrate solution was then added and the final absorbance read at 450 nm using the FLUOstar OPTIMA platereader (BMG Labtech Ltd., Aylesbury, UK). The results were analysed and calculated by the associated FLUOstar Optima Software V1.32.

In order to develop and validate an in-house assay for pCRP, several assay formats were investigated. Three of the formats will be described here starting with the successful assay. In all cases, the assay validation process was completed in a similar fashion unless preliminary results indicated that the assay was inferior and further investigation would be without merit.

2.2.3. Development of ELISA using Anti-Human C-Reactive Protein

A ligand-binding ELISA was developed for the measurement of CRP in serum. In this method, PC-BSA was coated onto microtitre plates and the CRP in diluted serum samples bound specifically to the PC-BSA coating. A rabbit anti-human CRP antibody (Sigma), assessed for cross-reactivity with porcine CRP by immunoblotting, was then applied. After washing, a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Abcam, Cambridge, UK) was applied and after washing, detected with use of tetra methyl benzidine substrate (KPL, Gaithersburg, MD, USA) .

2.2.3.1. Purification of Porcine CRP

Porcine CRP for use in assessing cross-reactivity was obtained from acute phase sera by affinity chromatography using the AKTA FPLC system (Amersham Biosciences, Uppsala, Sweden) running Unicorn v4 software. Sera was obtained and prepared as previously stated from pigs known to have tested positive for salmonella or *E. coli*. Acute phase sera were pooled and approximately 12 ml applied at 0.5 ml/min to a 5 ml immobilized *p*-Aminophenyl phosphoryl choline gel (Pierce Biotechnology, Rockford, Il. USA) equilibrated with 0.1 M Tris, 0.1 M NaCl (VWR International Ltd., Lutterworth, UK), 2 mM $CaCl_2$, pH 8-8.5. The column was washed with this binding buffer until the absorbance returned to baseline levels (approximately 12-15 column volumes). The bound CRP was eluted with 0.1 M Tris, 0.1 M NaCl (VWR International Ltd.), 2 mM EDTA (May & Baker Ltd, Dagenham, UK) pH 8-8.5 at 0.5ml/min. The CRP containing fraction was collected and buffer exchanged into 0.01 M sodium phosphate, 0.09 M NaCl (VWR International Ltd.) using centricon centrifugal filter devices (Millipore, Bedford, UK). This process was repeated in order to achieve a high purity of sample.

2.2.3.2. Gel Electrophoresis

The CRP containing fraction was assessed for homogeneity by SDS-PAGE, following reduction by mercaptoethanol. A 20 µl aliquot of CRP or serum (each aliquot was prepared from the same pool of CRP or serum throughout the development process) was mixed with 40 µl sample buffer reducing agent (3.55 ml dH_2O, 1.25 ml 0.5 M Tris, 2.5 ml glycerol, 2.0 ml 10% (w/v) SDS, 0.2 ml 0.5% (w/v) bromophenol blue) containing

5% v/v mercaptoethanol and heated for 5 min at 99°C. Aliquots of 20 µl of each sample and 10 µl of protein standards (Bio-Rad, UK) were applied to a SDS-PAGE gel (12% resolving, 4% stacking) and ran at 200V for approximately 30 to 45 min. The resulting gel was stained with Coomassie Brilliant Blue R-250 (500 ml methanol, 100 ml acetic acid, 400 ml dH_2O, 4 g Coomassie Blue Stain, mixed thoroughly and filtered) for 1 hr and destained in a solution of 75 ml acetic acid (VWR Chemicals, Leicestershire, UK), 200 ml methanol and 750 ml dH_2O.

2.2.3.3. Mass Spectrometry

To confirm the presence of porcine CRP, the relevant bands were cut out from the Coomassie Blue stained gel and were sent to The Sir Henry Wellcome Functional Genomics Facility at the University of Glasgow for mass spectrometry (MS) analysis. The bands were trypsin digested and the extracted peptides were desalted manually before using Q-Star Pulsar™ LC/MS/MS (Applied Biosystems, UK) for sequence analysis of the peptides. The resulting LC/MS/MS data were then searched in-house using Mascot™ software (www.matrixscience.com) equipped with translated databases (NCBI) for protein identification.

2.2.3.4. Immunoblotting

To confirm cross reactivity of anti-human CRP and porcine CRP, immunoblotting was performed. Proteins (including human CRP (Sigma)) were transferred from SDS-PAGE acrylamide gels onto nitrocellulose membranes (BioRad) at 100V for 1 hr using the Mini Trans-Blot cell (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, pH

8.3, 20% (v/v) methanol). Following protein transfer, the membranes were blocked with 1% (w/v) BSA in Tris buffered saline (TBS) with 1% v/v Tween-20, (100 mM Tris, 150 mM NaCl (VWR International Ltd.), pH 7.4 (TTBS)). The membranes were then incubated for 1 hr with rabbit anti-human C-reactive Protein (IgG fraction) (C3527, Sigma) diluted 1:1000 in TTBS, washed in TTBS and then incubated with HRP conjugated goat anti-rabbit IgG (Abcam, UK) diluted 1: 2000 in TTBS for 30 min. The membrane was washed with TTBS three times and developed using the Opti-4CN Substrate Kit (BioRad).

2.2.3.5. Conjugation of Phosphocholine to Bovine Serum Albumin

All reactions were carried out at room temperature unless otherwise stated and were based on the protocol of Eckersall *et al.* (1989a). Cytidine-5'-diphosphocholine (CDPC) (75 mg) in 0.5 ml 1 M NaOH, pH 8.4 was oxidised for 20 min in 2.5 ml 0.1 M sodium periodate (in 0.1 M NaOH, pH 8.4). Following oxidation, the remaining periodate was destroyed by addition of 0.15 ml 1M ethylene glycol. Bovine serum albumin (140 mg) was dissolved in 5 ml 0.01 M $NaHCO_3$, pH 9.6 and added to the activated CDPC. The pH of the reaction was maintained at pH 9.6 for 1 hr using 5% (w/v) Na_2CO_3, after which, reduction was performed overnight using 5 ml 0.5M sodium borohydride. The resulting solution was dialysed overnight at 4°C against 0.05 M Tris, pH 7.4, with 3 changes of buffer. To determine the outcome of the conjugation, the absorbance of the conjugate was measured at 280 nm and 270 nm using a Hitachi U-1500 Spectrophotometer with a successful conjugation shown by a shift in absorbance maximum from 280 nm to 270 nm due to the presence of conjugated PC-BSA.

2.2.3.6. Preparation of Standard Pool of Porcine Serum

A pool of porcine serum was made (n=10), and the pCRP concentration was determined by use of the Tridelta ELISA to be 70 mg/L. This pool was aliquoted and stored at -20°C until use as a standard in the pCRP assay.

2.2.3.7. Reagents and Buffers

The different buffers used throughout the assay are detailed below. The coating buffer was 0.01 M $NaHCO_3$, pH 9.4. The assay buffer was TBS-CT (0.05 M Tris, 0.15 M NaCl (VWR International Ltd.), 0.02 M $CaCl_2$, 0.1% Tween-20, pH 7.6); this buffer was made fresh every day. All buffers and reagents were applied at 200µl per microwell.

2.2.3.8. Assay Procedure

The assay conditions were optimised to achieve the best performance from the assay as described in section 2.3.3.9. The following protocol describes the final optimised conditions.

Coating

Two hundred microlitres of PC-BSA (diluted 1:400 in coating buffer) was applied to each microwell of a microtitre plate (Costar®, Corning Incorporated, Corning, NY, USA) and incubated at 4°C overnight. Coated plates could be prepared up to 3 days in advance.

Washing

The ELISA plate was washed by filling the wells with assay buffer, shaking the plate and decanting. This was repeated 3 times.

Blocking

Unoccupied binding sites were blocked with 250 µl of 1% (w/v) BSA in saline applied to each microwell and the plate incubated at 37°C for 1 hr. This was followed by washing in assay buffer as described.

Sample Dilution and Loading

Either sample or standard (200 µl) was applied in duplicate and incubated at 37°C for 1 hr followed by washing in assay buffer. The standards (equivalent to a range 0 – 70 mg/L) were prepared by diluting a pooled serum standard 1:1000 in assay buffer and serially diluting to give concentrations of 70, 35, 17.5, 8.75, 4.38 and 2.19 µg/L. Samples were diluted 1:2000 in assay buffer. Samples reading outside the range of the assay were re-assayed at an appropriate dilution. Internal controls of high, mid and low concentrations (determined previously by the commercial ELISA) were applied in duplicate and a blank (assay buffer only) applied in quadruplicate.

Primary Antibody Addition

Rabbit anti-human C-reactive Protein (IgG fraction) (Sigma) diluted 1:3000 in assay buffer was dispensed (200 µl) to each well and incubated at 37°C for 1 hr followed by 3x washing in assay buffer.

Secondary Antibody Addition

HRP conjugated goat anti-rabbit IgG (Abcam) diluted 1:5000 in assay buffer was applied (200 µl) and incubated at 37°C for 1 hr followed by 3x washing in assay buffer.

Substrate Addition

Tetra methyl benzidine (KPL) was prepared according to manufacturer instructions and 200 µl dispensed into each well. The ELISA plate was incubated at room temperature for approximately 5-7 minutes to allow the colour to develop. The enzyme reaction was stopped by the addition of 100 µl of 1 M HCl.

Absorbance

The absorbance was measured at 450 nm using the FLUOstar OPTIMA platereader (BMG Labtech Ltd.) and the results analysed and calculated by the associated FLUOstar Optima Software V1.32 R2 using a 4 Parameter fit standard curve plotted on a linear scale.

2.2.3.9. Assay Optimisation

Antibody Concentration

The optimal dilution of the antibody was determined by varying the antibody concentrations while keeping other variables constant. The aim was to identify the concentration of antibody that gave the maximum optical density (OD) for the highest standard and gave a straight standard curve, with low OD for the blanks. This was repeated using varying concentrations (1:2000 to 1:5000) of the primary antibody in order to find the optimal combination. The secondary antibody was optimised in a similar fashion

Optimisation of Standard and Sample Dilution

The standard pool was diluted at varying concentrations and then serially diluted in order to achieve standard curves over different ranges. The aim was to achieve a straight standard curve over a wide range of OD. Internal standards and samples were diluted at varying concentrations until they were able to be read on the linear section (0 – 35 mg/L) of the standard curve.

Blocking Agents

The ELISA was carried out using 5% (w/v) dried milk in saline or 1% (w/v) BSA in saline at the blocking step and the optical density for the blank samples were compared over 5 separate occasions.

2.2.3.10. Assay Validation

Specificity

Specificity was assessed by immunoblotting of purified pCRP and porcine acute phase sera as previously described in Section 2.2.3.4.

Precision

Intra-assay precision was determined by calculating coefficients of variation (CVs) of three samples with different CRP concentrations, replicated 4 times in the same assay run. To determine the inter-assay precision, CVs were calculated for the duplicates of the same serum samples over 21 runs during the use of the assay.

Accuracy

Accuracy was investigated by linearity under dilution of 3 serum samples with varying CRP concentration.

Limit of detection

The limit of detection was calculated as the lowest concentration of CRP which could be distinguished from a zero value, and was taken as the mean +2 standard deviations of 4 replicates of the blank samples.

2.2.3.11. Comparison to Other Methods

In order to confirm that the assay developed was comparable to the existing commercial assay, the concentration of CRP in 57 previously analysed (Tridelta ELISA) serum samples were also measured on the in-house ELISA. The results were compared using regression analysis.

2.2.4. Development of a Sandwich ELISA using Anti-Porcine CRP

2.2.4.1. Purification of Porcine CRP and Antibody Production

Purification of porcine CRP was carried out previous to this study by Dr F. M. Campbell (Faculty of Veterinary Medicine, University of Glasgow) and two antibodies produced; a polyclonal sheep anti-porcine CRP (BS/PTU, SNBTS, Penicuik, UK) and a chicken anti-porcine CRP (IgY antibody) (BS/PTU, SNBTS). These antibodies were made available for validation and use during this study.

2.2.4.2. Cross reactivity of Anti-Porcine CRP to Antigen

In order to test the reactivity of the antibody, gel electrophoresis of purified pCRP and porcine acute phase sera was carried out as previously described and the proteins transferred onto nitrocellulose membranes (BioRad) at 100V using the Mini Trans-Blot cell (Bio-Rad) in transfer buffer (25 mM Tris, 192 mM glycine, pH8.3, 20% (v/v) methanol). Following protein transfer, the membranes were blocked with 10% (w/v) non-fat milk in Tris buffered saline (TBS), (50 mM Tris, 100 mM NaCl (VWR International Ltd), pH 7.4) with 0.1% (v/v) Tween-20 (TTBS). The membranes were

incubated for 1 hr with either sheep anti-porcine CRP diluted 1:500 in TTBS or chicken anti-porcine CRP diluted 1:500 in TTBS , washed in TTBS and then incubated with HRP conjugated goat anti-sheep IgG (Abcam) diluted 1:1000 in TTBS for 30 min or HRP conjugated goat anti-chicken IgY (Abcam) diluted 1:1000 in TTBS for 30 min. The membrane was then washed with TTBS three times and developed using the Opti-4CN Substrate Kit (BioRad).

2.2.4.3. Antibody Processing

In order to remove non-specific antibodies from the antibodies to pCRP, the anti-serum was adsorbed against a solid phase reagent prepared from CRP depleted porcine serum by glutaraldehyde (Avrameas and Ternynck, 1969) prepared as below. This technique is based upon the covalent cross-linking of proteins with glutaraldehyde which provides specific and efficient immunoadsorbents for the purification of antibodies (or antigens).

2.2.4.4. Preparation of Immunoadsorbent

One millilitre of 1M acetate buffer pH 5 was added to 10ml of porcine CRP-depleted serum (the by-product of the purification of pCRP (Section 2.2.41). The pH of the solution was checked for acidity (< pH 5 desired) and if required, more buffer was added until <pH 5 was reached. Three millilitres of 2.5% aqueous glutaraldehyde was added drop wise to this solution while stirring gently and within 30 minutes a gel was formed. Gentle stirring continued for 3 hr at room temperature.

The resulting gel was mixed with 200 ml 0.2M phosphate buffer pH 7.2 – 7.4 (0.2 M $Na_2HPO_42H_2O$, 0.2 M $NaH_2PO_42H_2O$ mixed to pH) and homogenised (Ultra-turrax T25, Janke & Kunkel, IKA® Labortechnik, Germany) before being centrifuged at 1210 xg for 15 min at 4°C. The supernatant was removed and the process repeated one time. The gel was then mixed with 200 ml 0.1 M Glycine-HCl buffer pH 2.8, homogenised, centrifuged at 1210 xg for 5 min at 4°C and the supernatant removed. This was again repeated. The gel was then washed with 0.2 M phosphate buffer pH 7.2 – 7.4 and centrifuged at 1210 xg for 5 min at 4°C and the supernatant removed. The optical density (OD) of the supernatant was read at 280nm and the process repeated until the OD of the supernatant read 0. The gel was then immediately used or stored in phosphate buffered saline (PBS) at 4°C.

2.2.4.5. Use of Immunoadsorbent

To a volume of the immunoadsorbent gel, an equivalent volume of antibody was added. This solution was mixed overnight at 4°C and then centrifuged at 1210 xg for 15 minutes at 4°C. The supernatant (adsorbed anti-porcine CRP) was removed and stored for use. The immunoadsorbent was washed three times with PBS, two washes with 0.1M Glycine-HCl pH 2.8 and three washes with saline. The gel was then ready for further use or stored in PBS at 4°C.

Following adsorption, the specificity of the antibody was checked by gel electrophoresis and immunoblotting as described above.

2.2.4.6. Purification of Sheep Anti-Porcine CRP IgG Fraction

The IgG fraction was purified from the antiserum by affinity chromatography using an AKTA FPLC system (Amersham Biosciences) running Unicorn v4 software. Ten millilitres of the adsorbed antiserum was applied at 1 ml/min to a 5 ml HiTrap Protein G HP column (Amersham Biosciences) equilibrated with 20 mM sodium phosphate pH 7. The column was washed with this buffer until the absorbance returned to baseline levels. The bound IgG was eluted with 0.1 M Glycine-HCl, pH 2.7 at 5ml/min. The IgG containing fraction was collected, buffer exchanged and concentrated into TBS using Centricon centrifugal devices (Millipore) and stored in aliquots at -20°C. The specificity of the antibody was checked by immunoblotting as previously stated.

2.2.4.7. Purification and Processing of Chicken Anti-Porcine CRP IgY

Purification of chicken anti-porcine CRP IgY was carried out by Mrs M. Robinson (Faculty of Veterinary Medicine, University of Glasgow) using the Eggcellent™ Chicken IgY Purification Kit (Pierce, Rockford, IL. USA). The purified antibody was then processed as described in Section 2.3.4.5.

2.2.4.8. Biotinylation

The IgG fraction of the sheep anti pCRP antibody was then biotinylated. This process was carried out by Dr. L. Bence (Faculty of Veterinary Medicine, University of Glasgow) using an ImmunoProbe™ Biotinylation Kit (Sigma).

2.2.4.9. Preparation of Standard Pool

As previously stated in Section 2.3.3.6.

2.2.4.10. Reagents and Buffers

The different buffers used throughout the assay are detailed below. The coating buffer was 50 mM $NaHCO_3$, pH 9.6. The assay buffer was TTBS (50 mM Tris, 100 mM NaCl (VWR International Ltd.), 0.05% (v/v) Tween-20, pH 7.4). All buffers and reagents were applied at 100 µl per microwell.

2.2.4.11. Assay Procedure

The following protocol describes the best optimised conditions for this assay.

Adsorbed chicken anti-porcine CRP IgY (200µl), at a concentration of 500 ng/well, diluted in coating buffer was bound to microtitre plates (Maxisorp, Nunc, Denmark) and incubated for 2 hr at 37°C. The plate was then blocked with 250µl of 1% (w/v) BSA in assay buffer for 1 hr at 37°C. Following incubation, the plate was washed 3 times in assay buffer, and then standards or samples were applied for 1hr at 37°C. Following incubation, the plate was washed 3 times and biotinylated sheep anti-porcine CRP IgG was applied at 1:2000 (diluted in assay buffer) and the plate incubated for 1 hr at 37°C. Following washing, streptavidin peroxidase (Abcam) (1:1000 dilution) was applied and the plate incubated for 1 hr at 37 °C. Three washes in assay buffer followed and then freshly made TMB (KPL) applied. The colour was allowed to develop at room

temperature and the reaction stopped by the addition of 50 µl 1 M HCl. The absorbance was measured at 450 nm using the FLUOstar OPTIMA platereader (BMG Labtech Ltd.) and the results analysed by the associated FLUOstar Optima Software V1.32 R2 using a 4 Parameter fit standard curve plotted on a linear scale.

2.2.4.12. Assay Optimisation

Assay optimised as previously described in Section 2.2.3.9.

Antibody Concentration

This was carried out as previously described, by applying varying concentrations (1:2000 to 1:5000) of biotinylated anti-porcine CRP.

Optimisation of Standard and Sample Dilution

The standard pool was diluted at varying concentrations and then serially diluted in order to achieve standard curves over different ranges. Samples were diluted at varying concentrations until they were within the linear section of the standard curve.

Blocking Agents

This was carried out as previously described in Section 2.2.3.9.

2.2.4.13. Comparison to Other Methods

In order to confirm that the assay developed was comparable to the existing commercial assay, 17 serum samples which had previously been measured using the commercial assay were also measured on the ELISA. The correlation between the two CRP values was then analysed using Microsoft Excel.

2.2.5. Development of immunoturbidimetric assay

2.2.5.1. Antibody Preparation

Polyclonal sheep anti-porcine CRP was adsorbed as previously described in Section 2.3.4.5. and the IgG fraction purified as described in Section 2.2.4.6.

2.2.5.2. Preparation of Standards

Sera from animals with known high concentrations of CRP were pooled and the CRP concentration quantified using the Tridelta ELISA. The pool had a concentration of 125 mg/L. Standards over a range of 0 - 125 mg/L were then prepared by dilution of the pool in TBS (50 mM Tris, 150 mM NaCl (VWR International Ltd.), pH 7.4).

2.2.5.3. Assay Procedure

The following protocol describes the best optimised conditions for this assay on a Cobas MIRA analyser (Roche Diagnostics, Switzerland).

The analyser was programmed to mix 150µl of assay reagent (6% (w/v) polyethylene glycol in TBS) with 7.5µl of serum (standard or sample) in a reaction cuvette. Twenty five microlitres of antiserum was then added and the increase in absorbance at 340 nm was monitored every 25 seconds for a total of 25 readings. In order to calculate the concentration of CRP in samples, the change in absorbance from each sample was compared to the results obtained using the standard curve, with results reported in mg/L. To aid assay development, raw data was consulted showing the change in absorbance at each individual assay point within each sample or standard.

2.2.5.4. Assay Optimisation

Antibody

Both adsorbed sheep anti-porcine CRP antiserum and the IgG fraction of the antisera were utilised and the results compared.

Antibody Volume

A range of antibody volumes (25 -- 35 µl) were tested in order to find the optimal volume for the assay

Number of Readings

The number of readings (at 25 sec intervals) was varied from 25 to 35 until the optimal condition for the assay was found.

2.2.5.5. Comparison to other methods

In order to confirm that the assay developed was comparable to the existing commercial assay, the concentration of 15 serum samples which had previously been measured were also measured using this assay. The correlation between the two assays was then analysed.

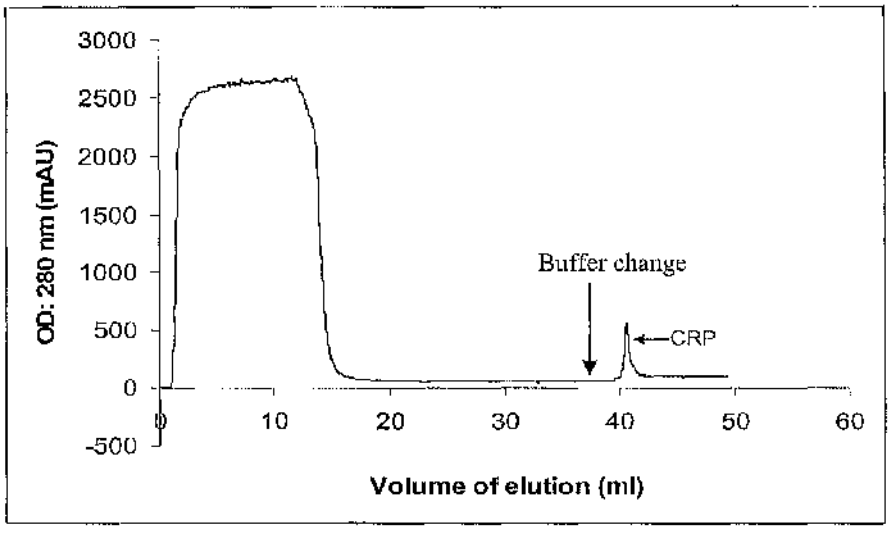
2.3.Results

2.3.1. Development of ELISA using Anti-human CRP

2.3.1.1. Purification of Porcine CRP

As shown by the chromatograms in Figure 2.2, the purification step on affinity chromatography yielded a distinct single peak eluting after the buffer changed to 0.1 M Tris, 0.1 M NaCl, 2 mM EDTA, pH 8-8.5, in both primary and repeat column runs. The eluted sample, as shown in Figure 2.3, consisted of 2 bands of 23 and 27 kDa and two bands at approximately 60 kDa and 110 kDa. The bands at 23 and 27 kDa were confirmed as porcine CRP after MS fingerprint data was examined using Mascot™ MS/MS Ions Search Analysis. This showed protein identity matches to accession number AB005546 (porcine CRP). See Appendix A for printout of the Mascot™ search. The 60kda band was identified as porcine serum albumin, the 110 kDa was not analysed at this time.

A



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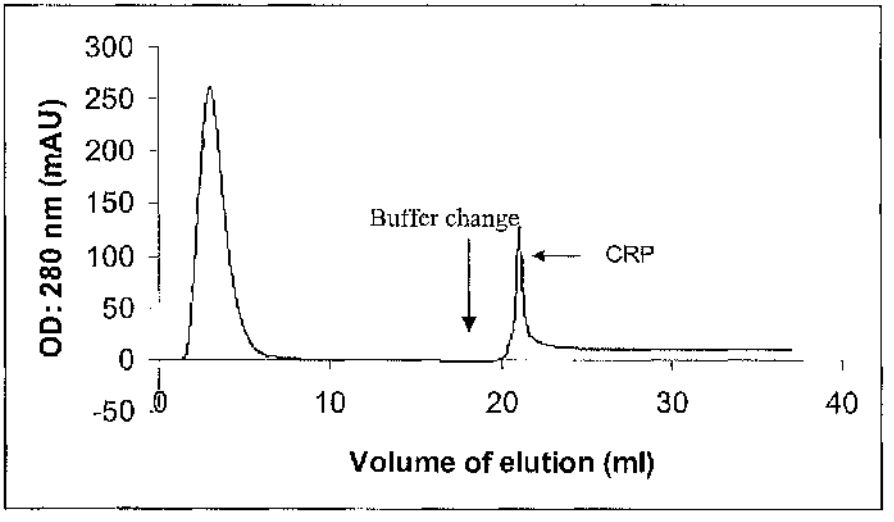


Figure 2.2: Elution profile of porcine CRP obtained from affinity chromatography. A: Primary purification of porcine CRP from serum using affinity chromatography. **B**: Repeated purification of porcine CRP from eluate obtained in A. Initial buffer was 0.1 M Tris, 0.1 M NaCl, 2 mM $CaCl_2$, pH 8-8.5 and was changed to 0.1 M Tris, 0.1 M NaCl, 2 mM EDTA, pH 8-8.5 as indicated.

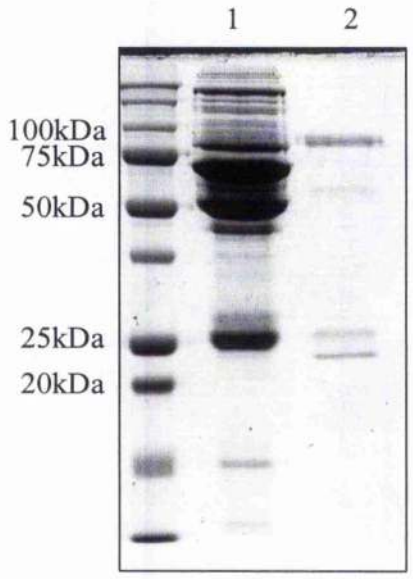


Figure 2.3: SDS-PAGE showing porcine acute phase serum (Lane 1) and purified porcine CRP after primary and repeat chromatography (Lane 2).

2.3.1.2. Cross Reactivity between anti human CRP and porcine CRP

Western blotting showed cross-reactivity between the porcine CRP band at 23 kDa and anti-human CRP (Figure 2.4) with a minor band at 20 kDa but no reaction with the 27 kDa, 60 kDa or the ~110 kDa bands on the gel. In addition, human CRP was run as a positive control and showed similar reactivity at 25 kDa.

2.3.1.3. Conjugation of phosphorylcholine to bovine serum albumin

After the conjugation there was a shift in absorbance maximum in the reaction from 0.484 at 280 nm to 0.532 at 270 nm. The concentration of PC-BSA stock solution was assessed as a relative value using the extinction co-efficient of BSA which is 0.67. The calculated value was 72.2. Future batches of the PC-BSA will be diluted to this concentration for future use.

2.3.1.4. Preparation of Standard Pool

The standard pool of serum was determined by the commercial assay to have a CRP concentration of 70 mg/L.

2.3.2. Assay Optimisation

During assay optimisation, graphical results are shown after the blank OD has been subtracted from the raw data, unless otherwise stated.

2.3.2.1. Antibody concentrations

During the optimisation process, a range of primary antibody concentrations were assessed, however, the final optimisation step compared the two dilutions; 1:2000 and 1:3000 as shown in Figure 2.5A. A final concentration of 1:3000 was chosen as it gave a reasonably steep curve and covered a wide range of OD values whilst giving lower blank readings than the 1:2000 dilution.

The secondary antibody was optimised in a similar fashion and the final optimisation process used 2 dilutions; 1:4000 and 1:5000 as shown in Figure 2.5. A secondary antibody concentration of 1:5000 was chosen as it resulted in a smoother curve, gave a good range of OD values and had lower blank readings. It also had a reasonable development time (~10 min) whilst the 1:4000 dilution developed overly fast (~3 min).

2.3.2.2. Optimisation of standard and sample dilution

The assay optimisation process required the preparation of a working standard curve. This was achieved by diluting the standard pool in assay buffer at a 1:1000 dilution and serial dilutions to give standards of 2.19 – 70 µg/L. An example of the standard curve is shown in Figure 2.6. Samples were diluted until they read accurately on the linear portion of the standard curve. Dependent upon the CRP content, this was at either 1:2000 or 1:4000 in assay buffer.

2.3.2.3. Blocking agents

The results of the blank samples with the different blocking agents are shown in Figure 2.7. Both blocking agents gave similar OD values however 1% BSA was chosen over

5% milk as it gave consistent values for OD whereas 5% milk showed some fluctuations.

During optimisation, each step was assessed by running samples which had previously been measured on the commercial assay. If there was little correlation between results of the two assays, the optimisation step was continued until the correlation was considered acceptable.

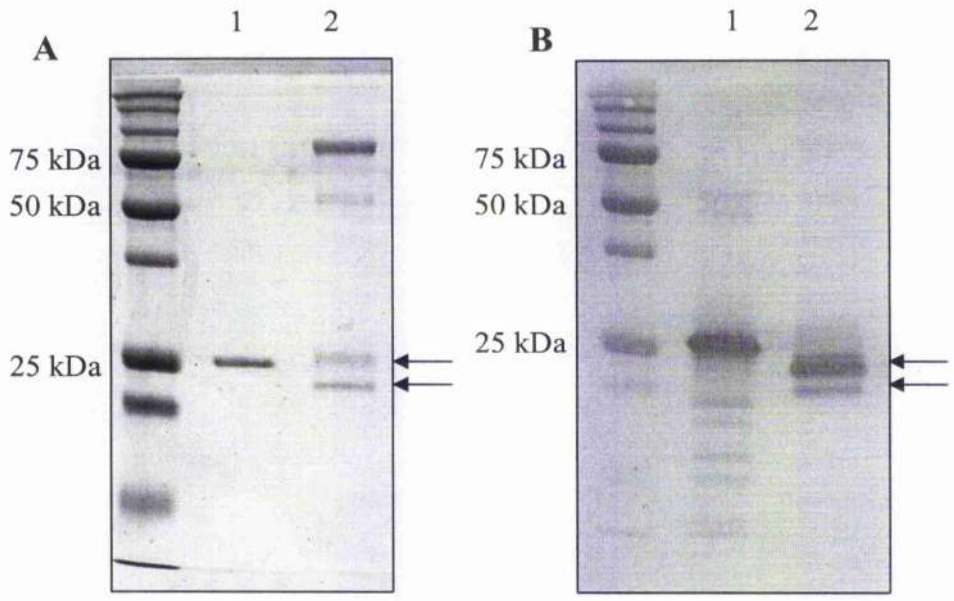
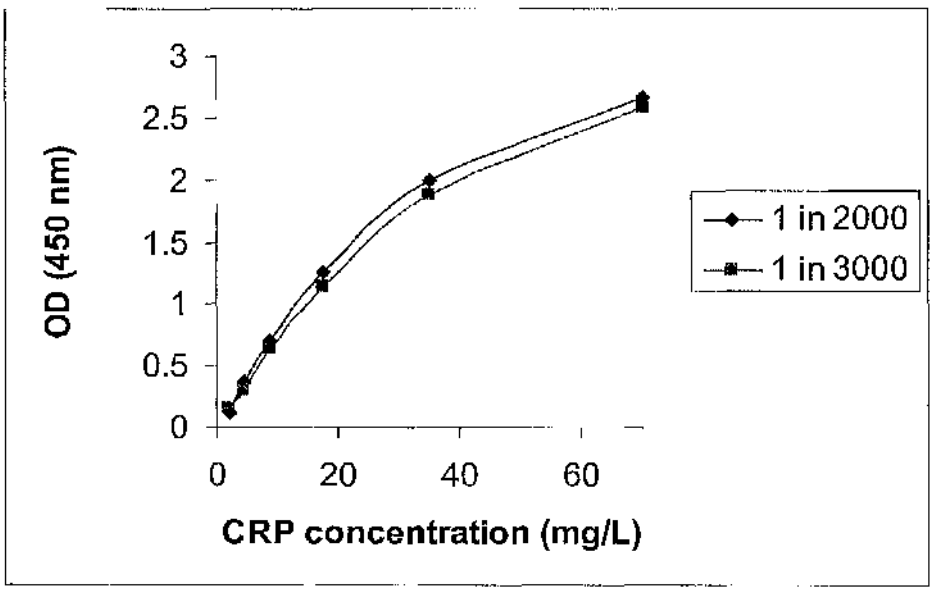


Figure 2.4: Cross reactivity of porcine CRP and anti-human CRP. A: SDS-PAGE gel of human CRP (Lane 1) and porcine CRP (Lane 2). Double arrows indicate position of porcine CRP at 27 and 23 kDa. **B:** Western blot with anti-human CRP showing reactivity with human CRP (Lane 1) and porcine CRP (Lane 2). Double arrows cross reactivity at 23 kDa and at a ~20 kDa band not visible on the SDS-PAGE gel. No cross-reactivity was seen with the 27 kDa band.

A



B

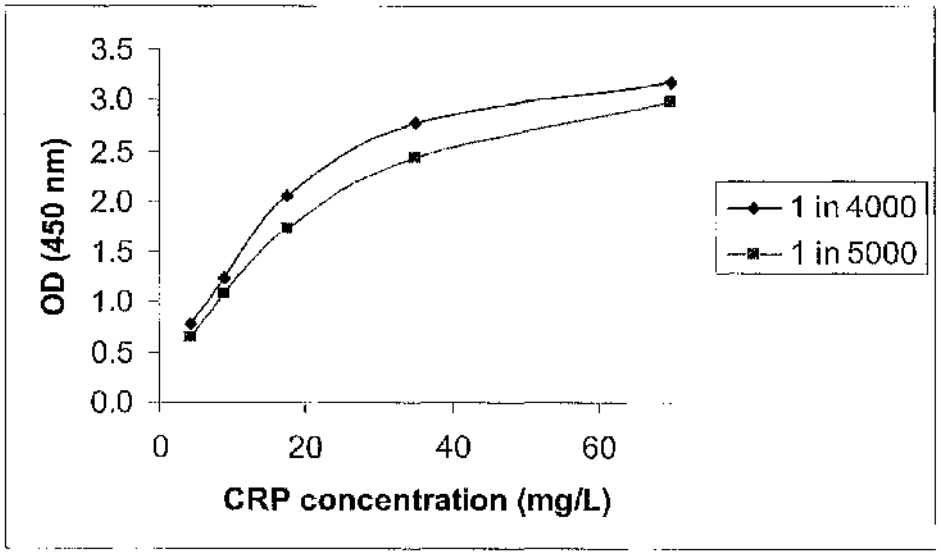


Figure 2.5: Optimisation of antibody concentrations for the developed ELISA. A: The effect of the anti-human CRP concentrations at 1:2000 and 1:3000. The 1:3000 dilution gave a range of OD values and lower blanks than 1:2000. **B:** The effect of the HRP conjugated antibody concentrations at 1:4000 and 1:5000. The 1:5000 dilution gave a range of OD values and lower blanks than 1:4000.

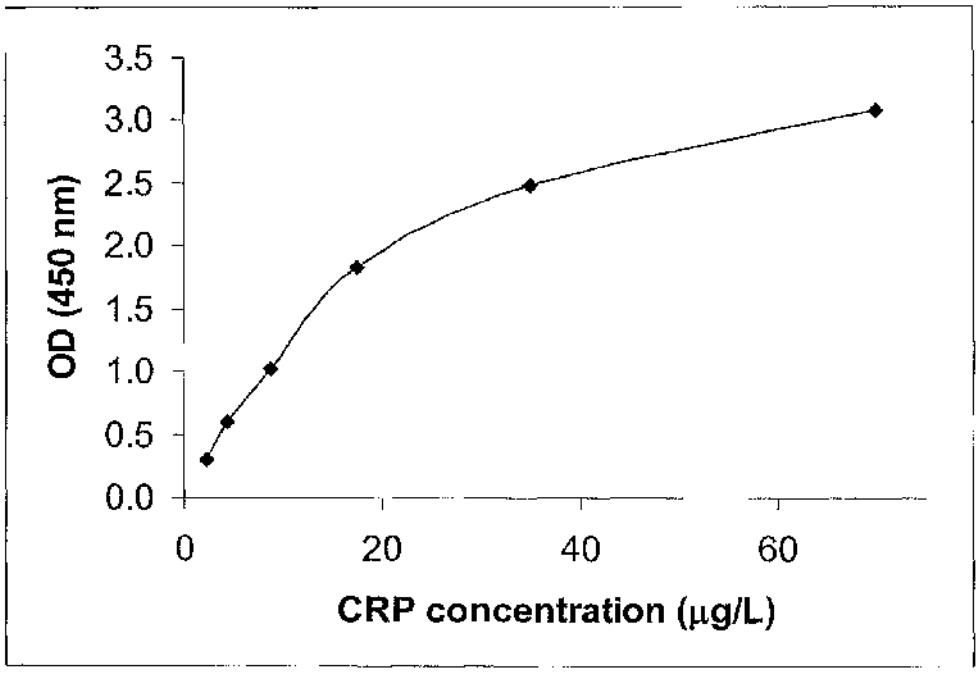


Figure 2.6: Standard curve obtained from optimised standard pool dilution. Standard pool diluted 1:1000 to 70 µg/L, then by serial 1:2 dilution.

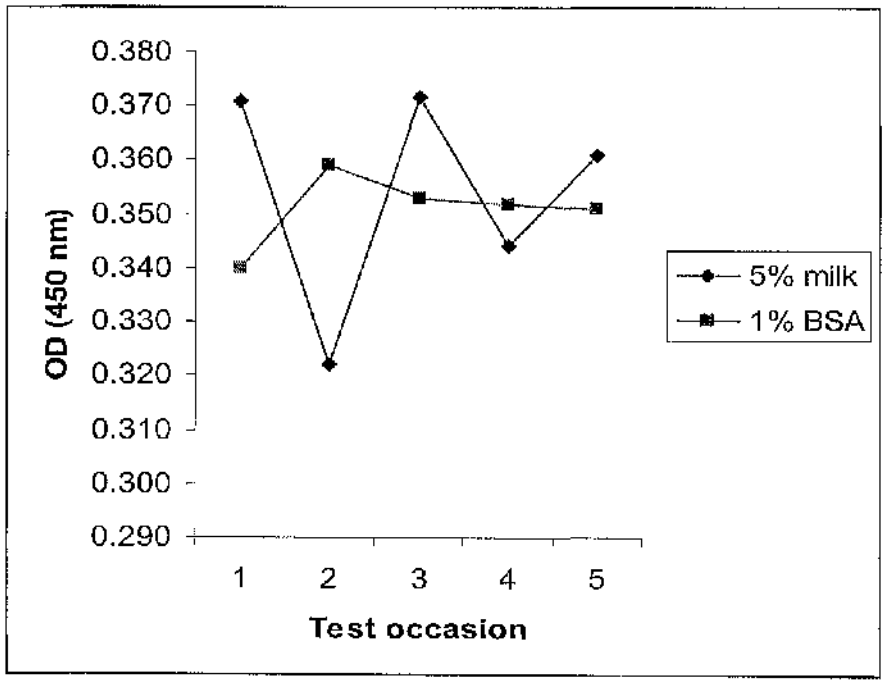


Figure 2.7: Effect of different blocking agents on the zero value of the blank samples on 5 separate occasions.

2.3.3. Assay Validation

2.3.3.1. Precision

The intra-assay CVs were in the range of 1.98 – 11.63% and inter-assay CVs were in the range of 5.75 – 10.43%, showing acceptable precision (Table 2.1).

2.3.3.2. Accuracy

Accuracy was investigated by serial dilution of 3 serum samples and is shown in Figure 2.8. The three samples show linearity then plateau out at low dilution and are parallel to the standards. Diluted sample concentrations were taken from the linear portion of the curve (this is the most precise and accurate area of the curve).

2.3.3.3. Limit of detection

The minimum detection limit significantly different from the blank samples was taken as the CRP concentration at 2 standard deviations (SD) from the mean of blank sample (n=4). This resulted in a limit of detection of CRP in serum of 0.016 mg/L.

Table 2.1: Inter- and intra-assay precision results obtained for ELISA with serum samples.

	n	**Intra-assay**		*n*	**Inter-assay**	
		CRP (mg/L) ±SD	CV		CRP (mg/L) ±SD	CV
Sample 1	4	240 ± 5	1.98	21	236 ± 14	5.75
Sample 2	4	565 ± 66	11.63	21	446 ± 47	10.43
Sample 3	4	109 ± 4	3.22	21	105 ± 6	6.13

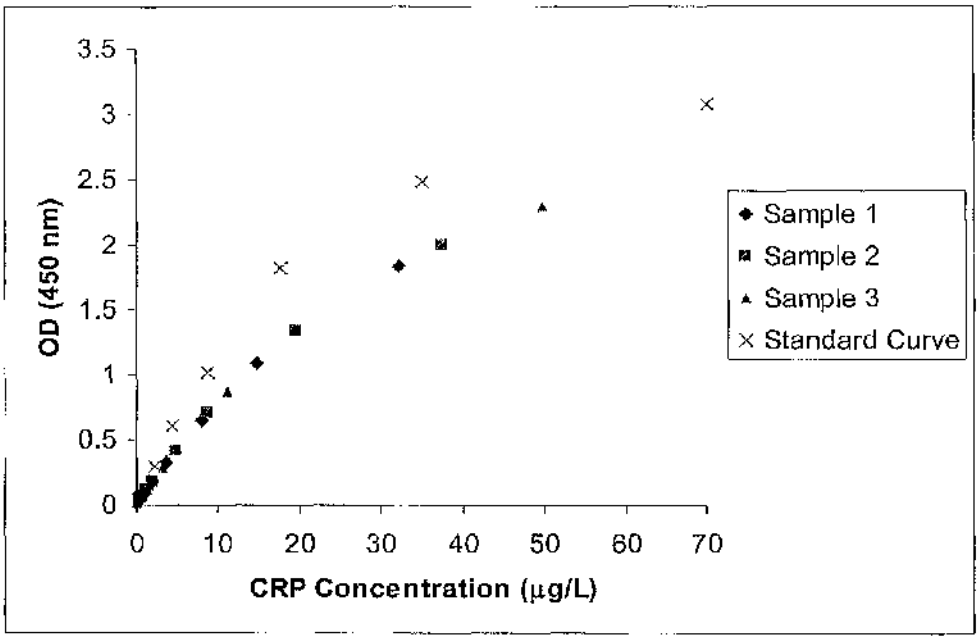


Figure 2.8: Linearity of 3 porcine serum samples after serial dilution. Example of standard curve included for comparison.

2.3.3.4. Comparison to other methods

The concentrations of CRP in the serum samples measured by both the commercial assay and the developed assay are shown in Figure 2.9. A total of 57 serum samples were measured by both assays and the comparison between them revealed a significant correlation of $r = 0.841$ ($p<0.001$) and regression equation of $y=1.1495x + 11.146$.

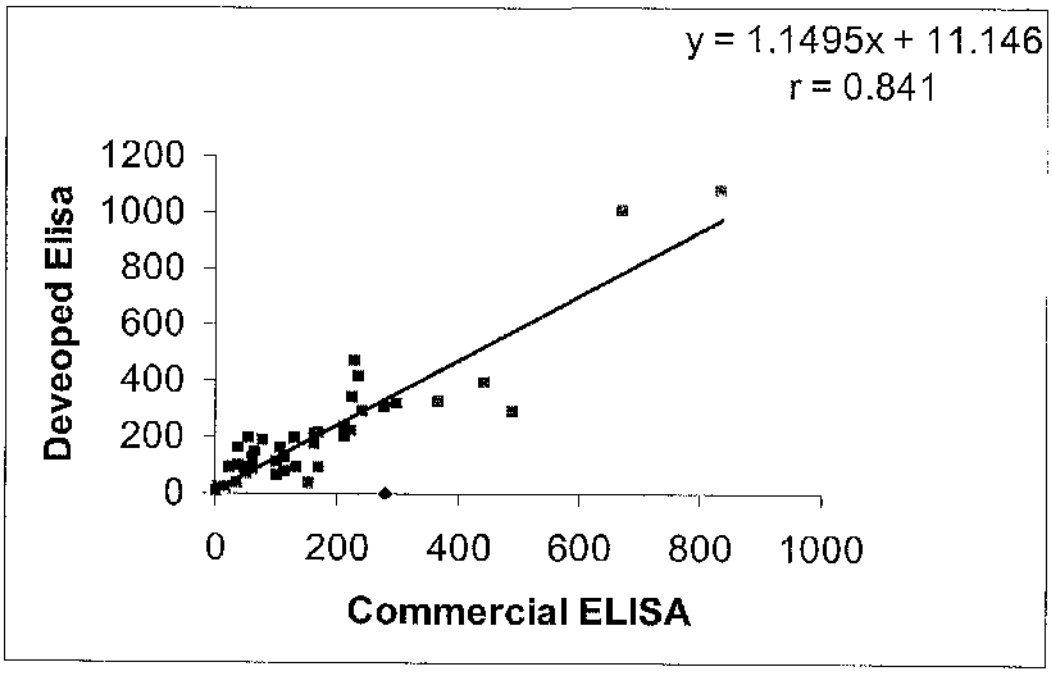


Figure 2.9: Correlation between measurements of CRP in 57 serum samples using a commercial ELISA (Phase Range™ Porcine C-reactive protein assay, Tridelta Development Plc., Ireland) (horizontal axis) and the developed ELISA (vertical axis).

2.3.4. Development of Sandwich ELISA

2.3.4.1. Adsorption of Sheep Anti-porcine CRP with Depleted Serum

Sheep anti-porcine CRP was adsorbed against depleted serum (as described in Section 2.3.4.3), the cross-reactivity of the adsorbed antisera was assessed by western blot as demonstrated in Figure 2.10.

2.3.4.2. Preparation of IgG Fraction of Sheep Anti-porcine CRP

As shown by the chromatogram in Figure 2.11, the purification of IgG on a Protein G column yielded a distinct single peak. The specificity of this antibody was then checked against pCRP and porcine acute phase sera using SDS-PAGE and western blotting as shown in Figure 2.10.

2.3.4.3. Cross Reactivity of Sheep Anti-porcine CRP to Porcine CRP

Western blotting showed cross-reactivity between all three fractions of the sheep anti-porcine CRP (anti-CRP, adsorbed anti-CRP and adsorbed anti-CRP IgG and porcine CRP) as shown in Figure 2.10. Cross-reactivity can be seen against the pCRP bands and 2 bands at ~ 50 and 75 kDa. The IgG fraction was then chosen for biotinylation and use as a signal antibody in the sandwich ELISA assay.

2.3.4.4. Cross reactivity of chicken anti-porcine CRP to porcine CRP

Western blotting showed cross-reactivity between the two fractions of the chicken anti-porcine CRP; anti-CRP and adsorbed anti-CRP as shown in Figure 2.12. Reactivity was seen against the pCRP bands at 23 and 27kDa and bands at ~ 50 and 75 kDa. The specificity of the anti-serum can be seen to increase with the processing steps and the adsorbed fraction was chosen for use as capture antibody in the sandwich ELISA assay.

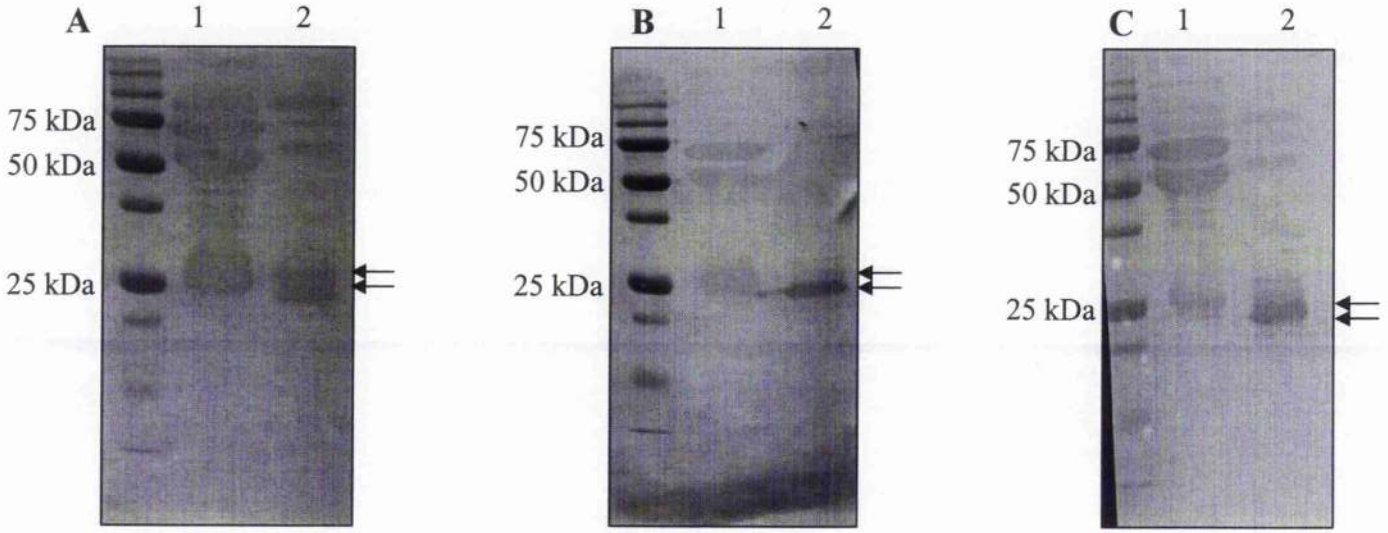


Figure 2.10: Cross-reactivity between sheep anti-CRP fractions and porcine acute phase serum (Lane 1) and porcine CRP (Lane 2). A: Western blot with sheep anti-porcine CRP. **B**: Western blot with adsorbed sheep anti-porcine CRP. **C**: Western blot with adsorbed sheep anti-porcine CRP IgG. CRP bands are indicated by double arrows (27 kDa and 23 kDa).

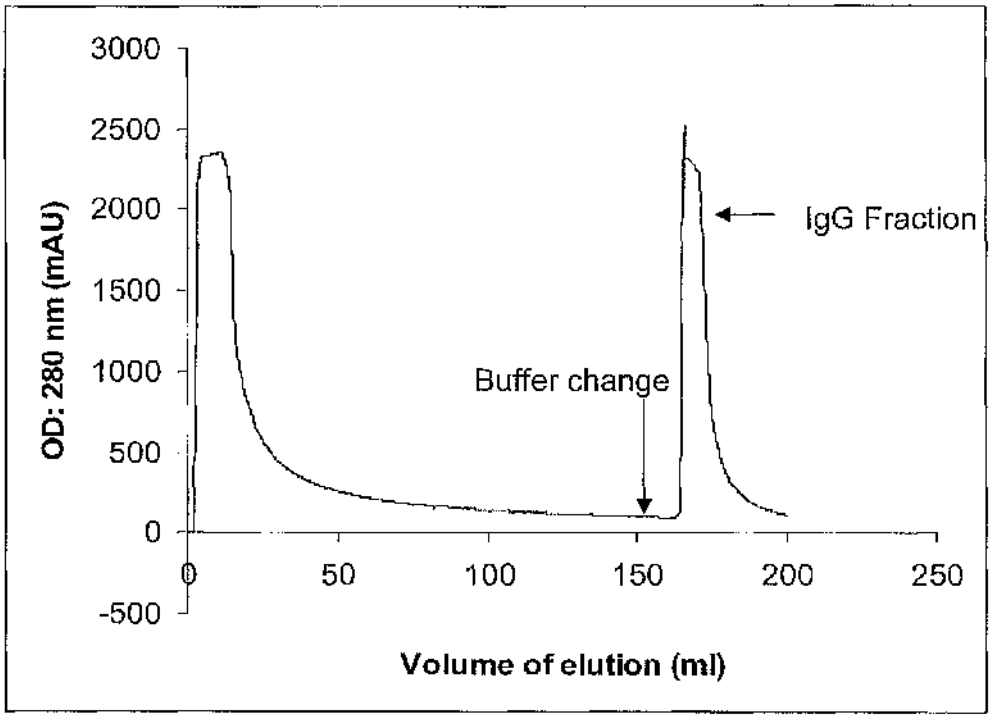


Figure 2.11: Elution profile of sheep anti-porcine CRP IgG obtained from affinity chromatography on a Protein G HP column.

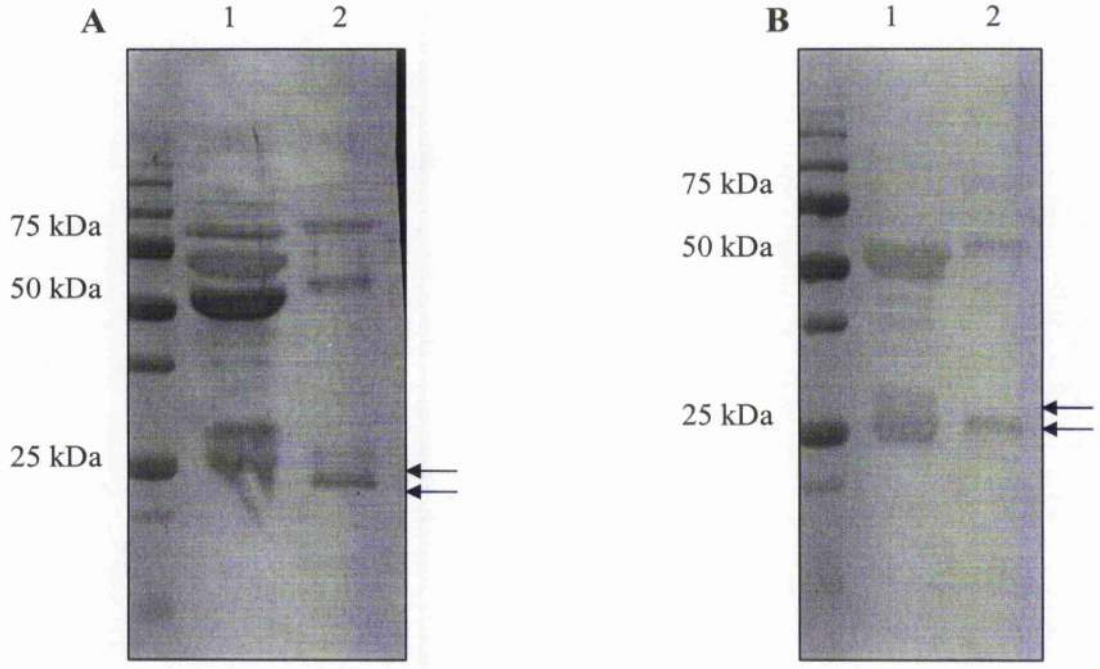


Figure 2.12: Cross reactivity between chicken anti-porcine CRP fractions and porcine acute phase serum (Lane 1) and porcine CRP (Lane 2). A: Western blot with chicken anti-porcine CRP. **B:** Western blot with adsorbed chicken anti-porcine CRP. CRP bands are indicated by double arrows at 27 kDa and 23 kDa.

2.3.4.5. Assay Optimisation

Antibody Concentration

During the optimisation process, a range of biotinylated anti-porcine CRP IgG concentrations were used. The two most effective, 1:2000 and 1:5000 are shown in Figure 2.13. The 1:2000 dilution was selected as it gave a wider range of OD values and gave a reasonably steep curve when compared to the 1:5000 dilution.

Optimisation of Standard and Sample Dilution

In order to achieve a working standard curve, a pool of serum standard was diluted in assay buffer at a 1:1000 dilution. An example of a standard curve is shown in Figure 2.14. Samples were diluted until they could be read on the linear portion of the curve.

Blocking Agents

The results of the blank samples with the different blocking agents are shown in Figure 2.15. 1% BSA was chosen over the 5 % milk as it gave consistently lower OD values.

2.3.4.6. Comparison to other methods

The concentrations of CRP in the serum samples measured by both the Tridelta ELISA and the developed sandwich ELISA are shown in Figure 2.16. An outlier was apparent (at ~1000 mg/L by the commercial ELISA), however considering the high concentration it is possible that the measurement by the commercial ELISA is inaccurate, the value was left in the analysis for a true representation. The comparison between them revealed a low correlation of r=0.44 and a regression equation of y=0.2036x.

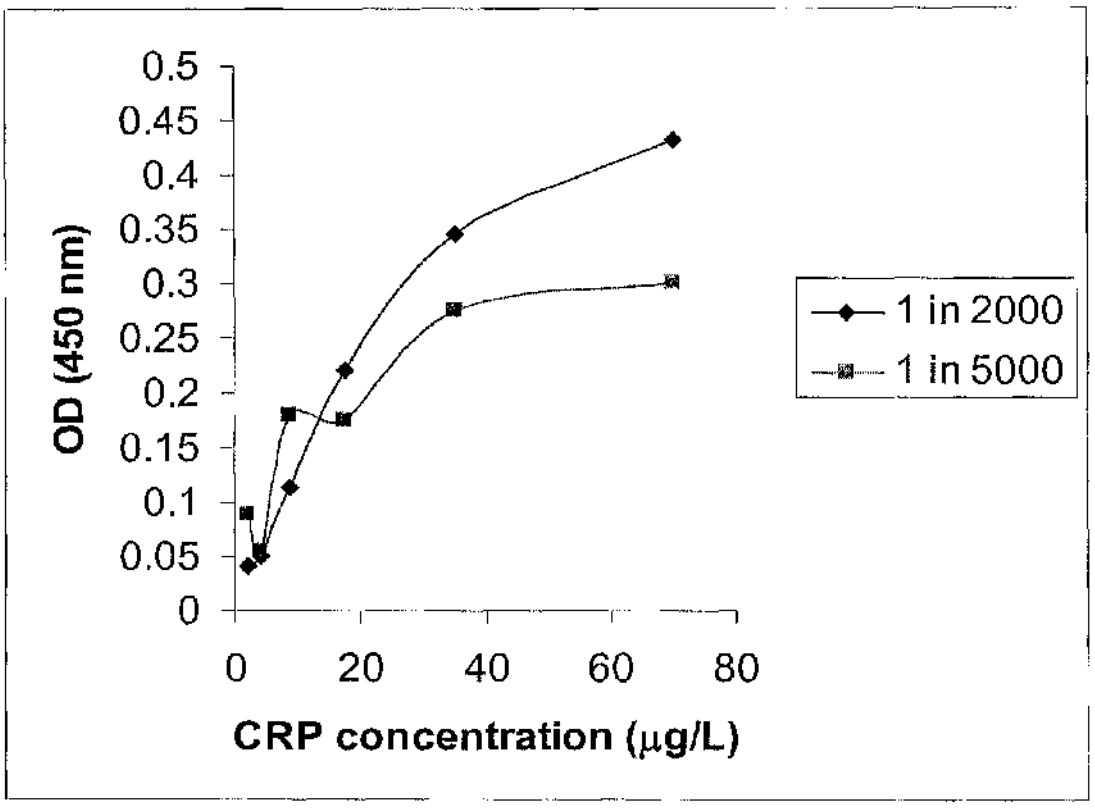


Figure 2.13: Optimisation of antibody concentrations for the developed ELISA. The effect of biotinylated sheep anti-porcine CRP IgG concentrations at 1:2000 and 1:5000. The 1:2000 dilution gave a wider range of OD values and a smoother curve than the 1:5000 dilution.

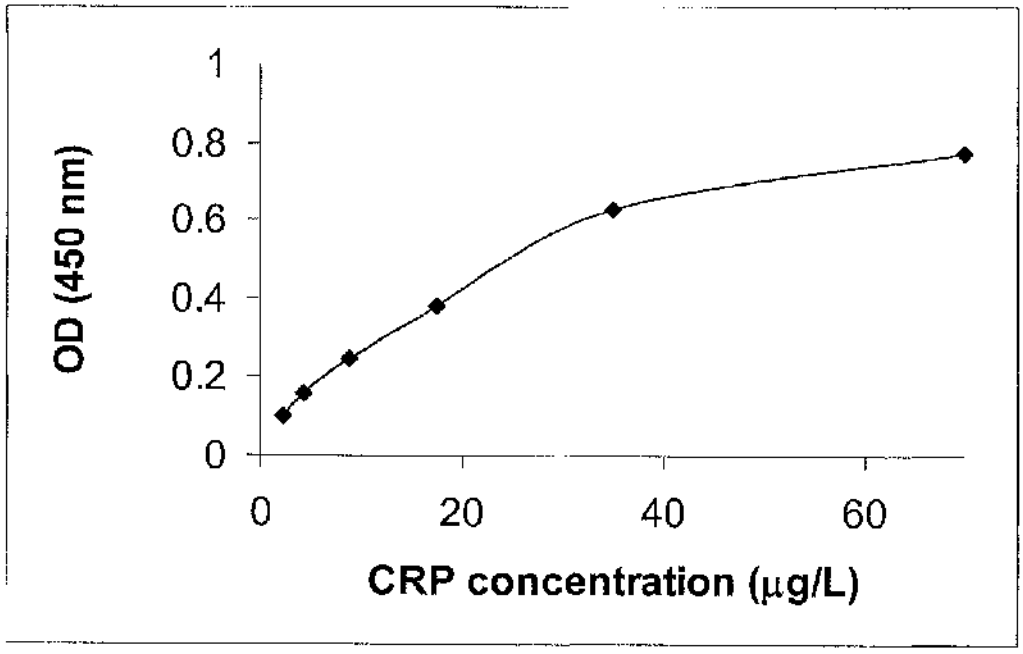


Figure 2.14: An example of a standard curve obtained from optimised standard pool dilution. Standard pool diluted 1:1000, then by serial 1:2 dilution.

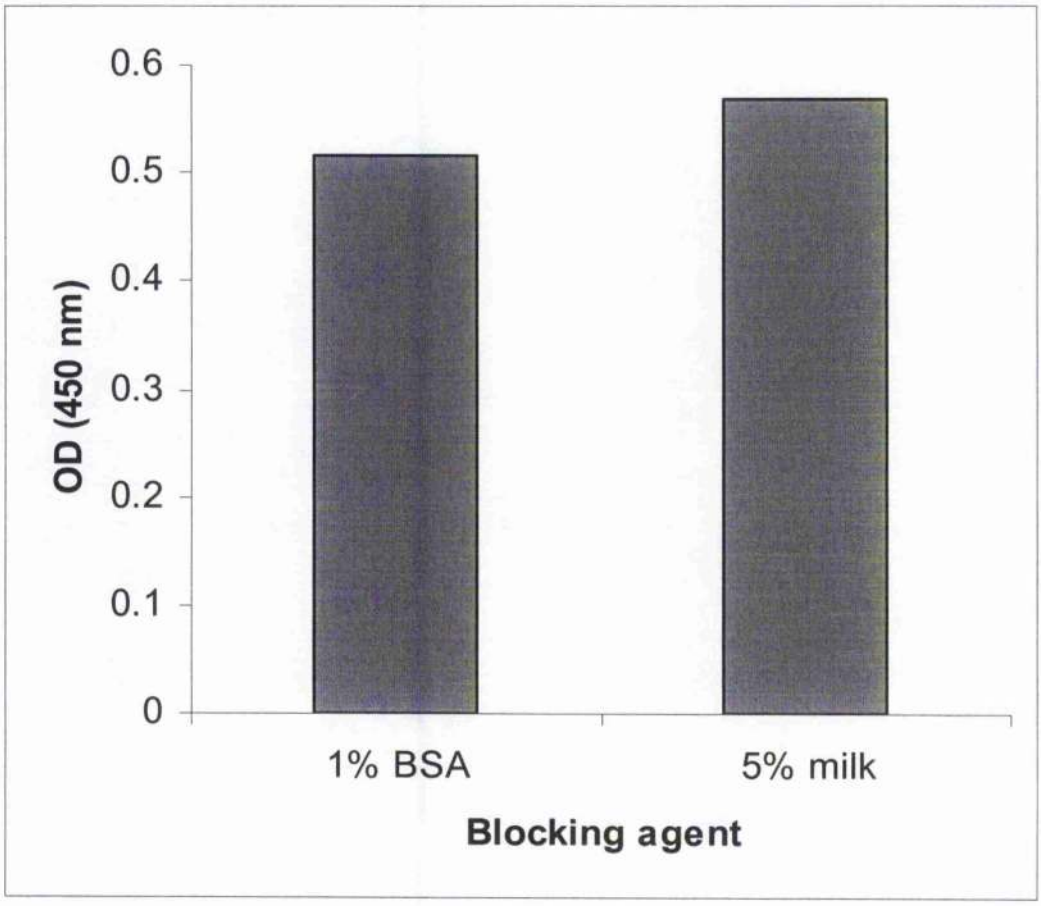


Figure 2.15: Effect of different blocking agents on the zero value of the blank samples.

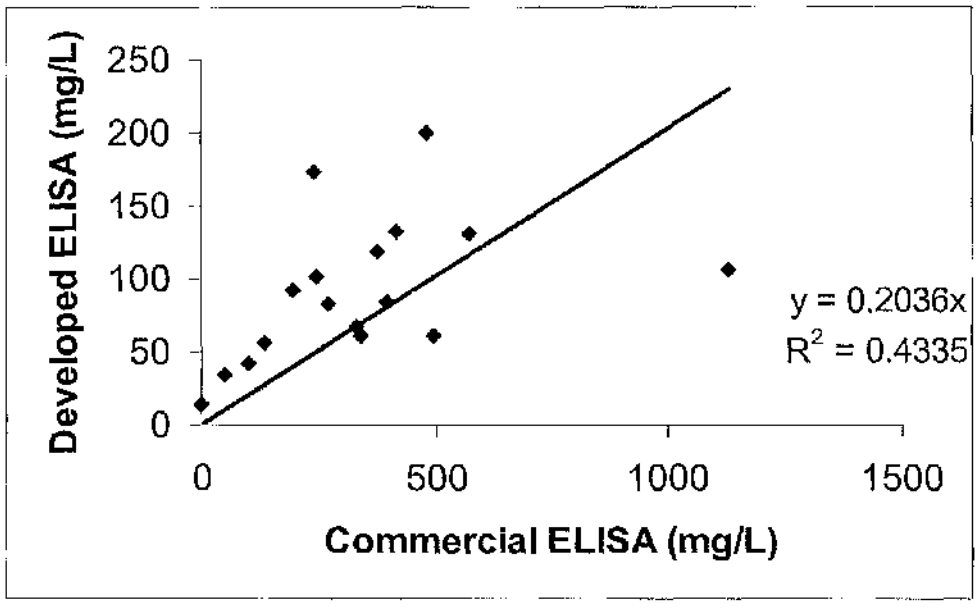


Figure 2.16: Correlation between measurements of CRP in serum samples using a commercial ELISA (Phase Range™ Porcine C-reactive protein assay, Tridelta Development Plc., Ireland) (horizontal axis) and the developed ELISA (vertical axis).

2.3.5. Development of an Immunoturbidimetric Assay

2.3.5.1. Assay Optimisation

Antibody Choice

The two antibody preparations selected for this assay; adsorbed sheep anti-porcine CRP and sheep adsorbed anti-porcine CRP IgG, were compared. The effect of these two antibodies on the calibration curve obtained on the MIRA analyser is shown in Figure 2.17. The adsorbed Anti-CRP was chosen for use and further development as it had much higher rate of reactions with a max of 0.19 (Figure 2.17B) than the IgG fraction with a max of 0.04 (Figure 2.17A).

Antibody Volume

The effect of changing the antibody volume in the reaction is shown in Figure 2.18. Similar results were obtained for the 3 volumes tested; however, a volume of 25 µl was selected as it gave a linear calibration curve and prevented wastage of antibody.

Reaction Time

The effect of changing the reaction time by adjusting the number of readings in each reaction during the assay is shown in Figure 2.19. Twenty-five readings (625 sec) were chosen for use as this gave a linear calibration curve with a shorter assay time.

Limit of Detection

Blank samples (assay buffer) were assessed in each assay run. However, a value of >0.08 mg/L was frequently given for these samples and low pCRP concentrations (<5 mg/L) were unable to be determined accurately.

2.3.5.2. Comparison with other methods

The concentrations of CRP in the serum samples measured by both the Tridelta ELISA and the developed immunoturbidimetric assay are shown in Figure 2.20. Two outliers were apparent measured at approximately 25 and 55 mg/L by the commercial assay, however the immunoturbidimetric assay consistently measured them higher. These were left in the correlation analysis to represent the true nature of the assays. The comparison between them revealed a correlation of r=0.62 and regression equation of y=1.0268x + 10.413.

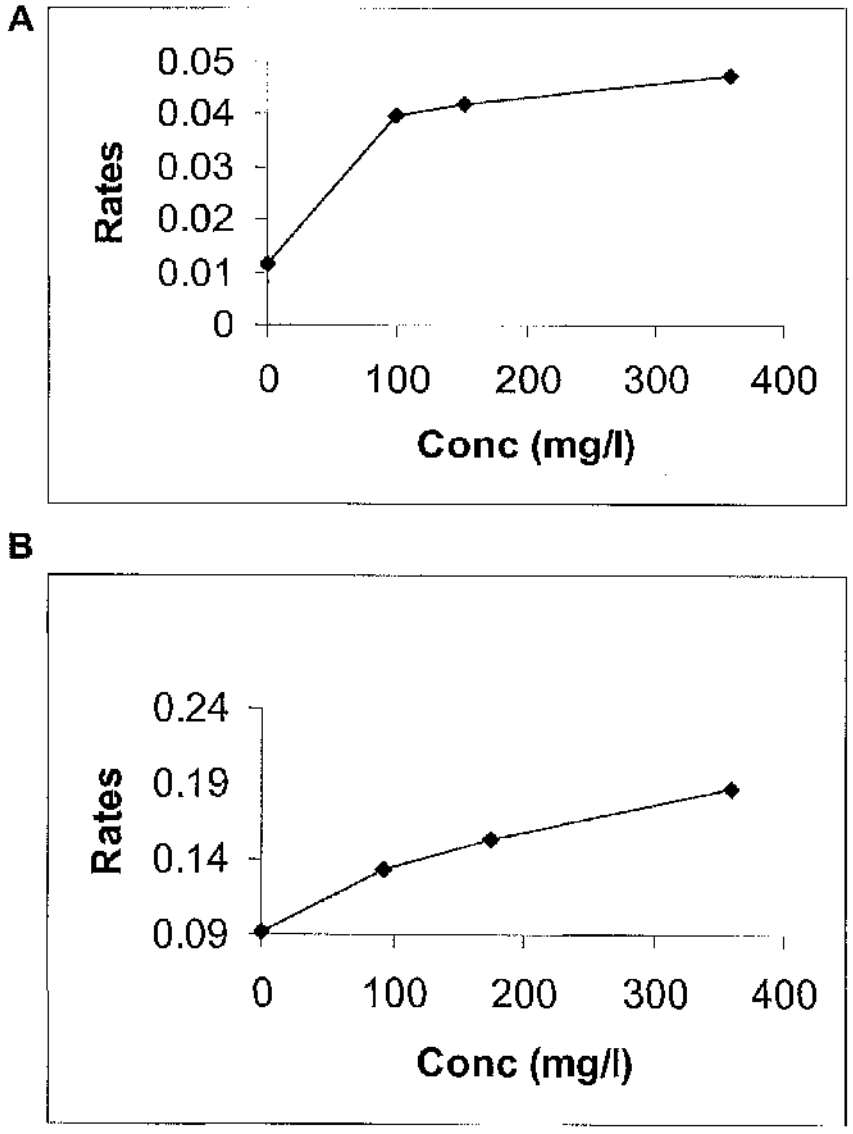
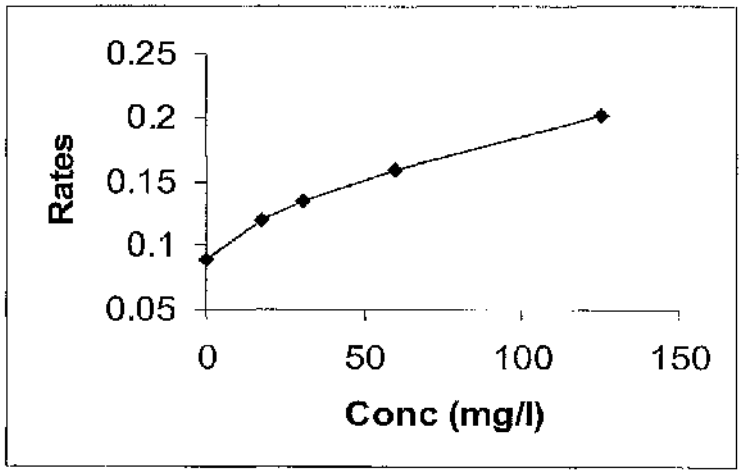


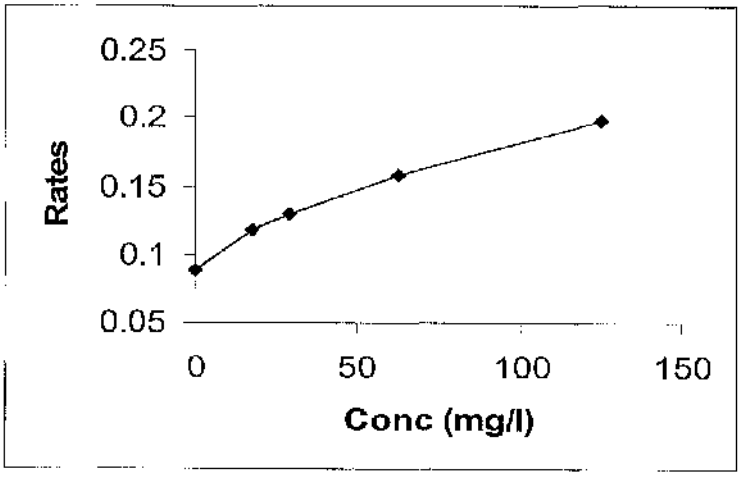
Figure 2.17: Comparison of antibodies for use in immunoturbidimetric assay. A: Calibration curve when sheep anti-porcine CRP IgG used in assay. **B:** Calibration curve when using adsorbed sheep anti-porcine CRP in assay. **Note difference in scale of y-axis.**

Figure 2.18: Comparison of antibody volumes for use in immunoturbidimetric assay.

A



B



C

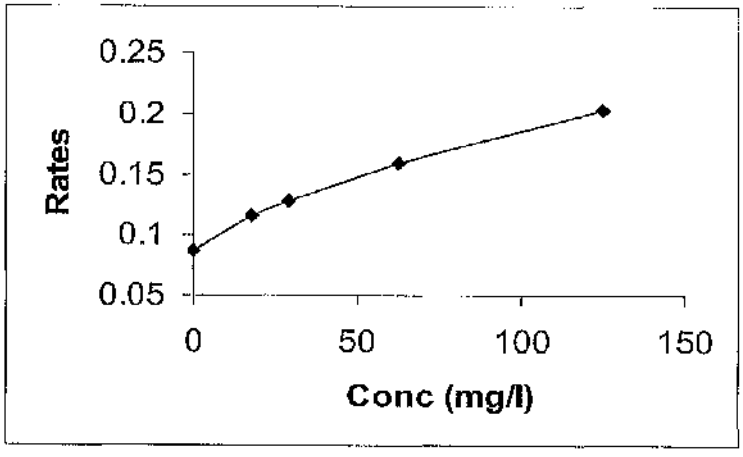
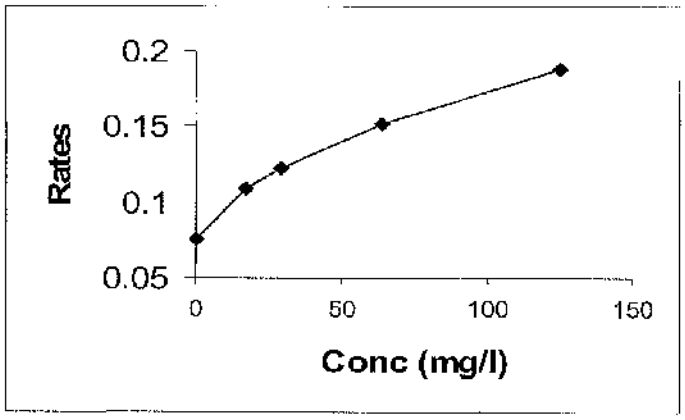


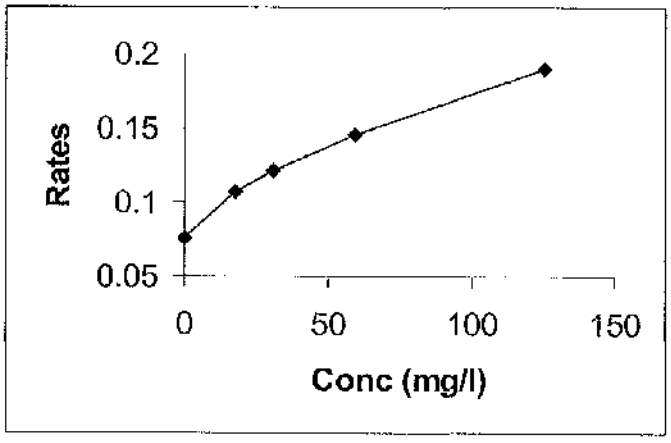
Figure 2.18: Comparison of antibody volumes for use in immunoturbidimetric assay. A: Calibration curve when 35 µl of antibody used. **B:** Calibration curve when 30 µl of antibody used. **C:** Calibration curve when 25 µl of antibody used.

Figure 2.19: Comparison of the number of readings during reaction.

A



B



C

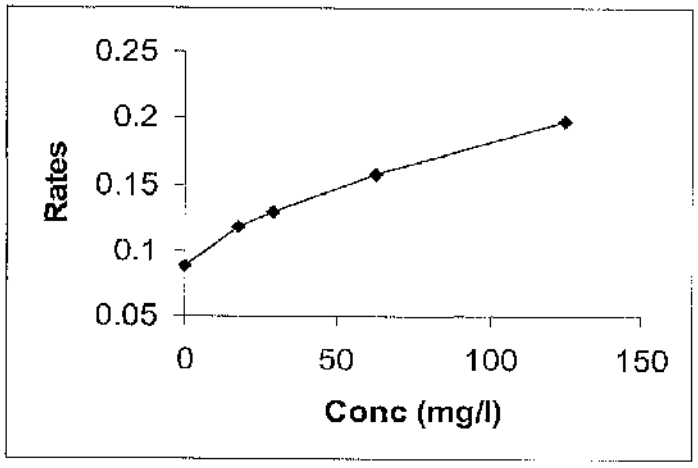


Figure 2.19: Comparison of the number of readings during reaction. A: Calibration curve showing effect of 25 readings. **B:** Calibration curve showing effect of 30 readings. **C:** Calibration curve showing effect of 35 readings. Changing the number of readings had little effect on rate of reaction.

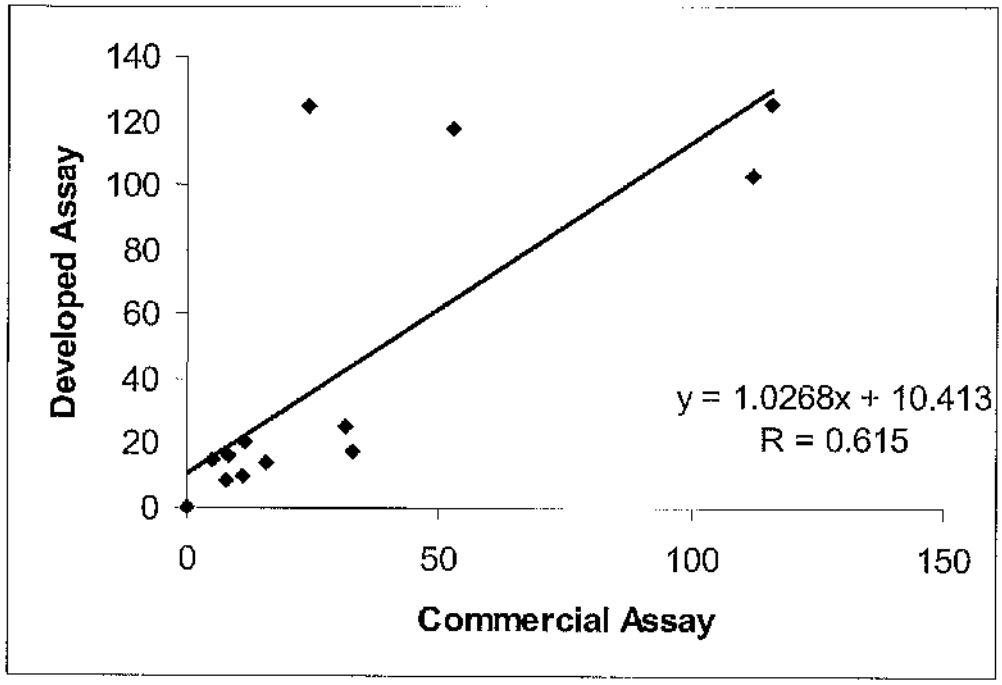


Figure 2.20: Correlation between simultaneous measurements of CRP in serum samples using a commercial ELISA (Phase Range™ Porcine C-reactive protein assay; (horizontal axis) and the developed assay (vertical axis).

2.4.Discussion

C-reactive protein is considered a major APP in pigs and is frequently measured in health studies (Petersen *et al.*, 2001). A commercial assay has been validated for use in the measurement of porcine CRP but the use of commercial assays can prove costly and the results may be imprecise, particularly at low concentrations (Tecles *et al.*, 2007). Various 'in-house' assays have been developed including ELISA and immunoturbidimetric assays and have been validated for use (Sorensen *et al.*, 2006, Kjelgaard-Hansen *et al.*, 2003). A number of these assays are based upon the cross reactivity of porcine CRP with human anti-CRP. The aim of this study was to develop an in-house ELISA for the measurement of porcine CRP in serum which would later be used in a study of a commercial pig population.

Previous to this study, porcine CRP had been purified and two polyclonal antibodies produced; a sheep anti-porcine CRP and a chicken anti-porcine CRP. Initial results revealed a high amount of non-specificity in both antibodies when tested against acute phase sera and CRP by immunoblotting. Processing of the antibodies by using a solid phase porcine serum depleted of CRP removed some of the non-specific reaction, and in the case of the adsorbed sheep anti-porcine CRP, an IgG fraction was purified from the adsorbed antisera using a Protein G HP column, which also improved specificity. Immunoblotting still showed slight background reactivity using the adsorbed anti-porcine CRP IgG against acute phase porcine serum and in particular a band was present at ~105-115kDa. However, as the pCRP bands appeared stronger in the western blot than the non-specific binding, the antibody was considered suitable for use, particularly in the sandwich ELISA where the specificity of 2 antibodies was combined.

Two assay formats were initially developed with these antisera; an immunoturbidimetric assay and a sandwich ELISA. The immunoturbidimetric assay which would have the advantage of being an automated assay, used the sheep anti-porcine CRP. Initial optimisation showed that the absorbed antibody gave higher rates of reaction than the absorbed IgG fraction and this was further optimised with regards to number of readings and volume of antibody added to the reaction mixture. A small number of samples were tested on this assay (n=15) and the initial correlation was r=0.62. Although further work could have improved the assay its major disadvantage assay was its inability to detect pCRP at low concentrations (<5 mg/L) thus lower values of CRP, which are expected in healthy pigs (for example, the commercial herd analysed in chapter 3 had CRP concentrations ≥0.9 mg/L), could not be read accurately by the analyser. As a result a different assay format was tried.

The sandwich ELISA used both the sheep anti-porcine CRP IgG and a biotinylated aliquot of the adsorbed chicken anti-porcine CRP. Optimisation of the protocol led to a working assay. The assay gave a low correlation (r= 0.44) to CRP concentrations determined by the Tridelta assay and on closer inspection, some values varied greatly from the previous results. These discrepancies could be attributed to two factors; a) the Tridelta ELISA was inaccurate or b) the antibodies were showing non-specific reactions. The Tridelta ELISA has been used in the calibration of assays previous to this study (Martinez-Subiela *et al.*, 2007, Sorensen *et al.*, 2006), and as a result it was deemed that the antibody specificity may have been the factor at fault and development of this assay was halted.

Porcine CRP shows considerable homology to human CRP and cross-reactivity between porcine CRP and anti-human CRP has been shown previously (Kjelgaard-Hansen *et al.*, 2003, Heegaard *et al.*, 1998, Eckersall *et al.*, 1996). Porcine CRP was purified from serum and revealed four distinct bands on SDS-PAGE gels. The bands at 23 and 27 kDa have been reported previously (Martinez-Subiela *et al.*, 2007) and mass spectrometry analysis confirmed porcine CRP identity. The third band was identified as serum albumin which has not been removed during the purification process. The fourth band (~105-115kDa) has not been described in published papers but may be unreduced CRP (the theoretical mass of CRP is 117 kDa) or an aggregate; however this has not yet been confirmed by mass spectrometry. The molecular weight of this band does correspond to the pentameric structure of the protein. If this were the case, it would partially explain the background reactivity observed in the sheep and chicken anti-porcine CRP antisera. Immunoblotting with anti-human CRP revealed cross-reactivity with the CRP band at 23 kDa and was used as the basis of a ligand binding ELISA. Differences in the CRP band patterns and cross-reactivity could be due to changes in the glycosylation of CRP as this has been reported in humans (Das *et al.*, 2003) but as yet, no research has been carried out in pigs. A band at 20 kDa also showed cross-reactivity with anti-human CRP which was not visually apparent on SDS-PAGE gels. The identity of this band is unknown and has not been previously described in published papers but may be a degradation product.

PC-BSA binds to CRP and is produced in a simple reaction, however it should be noted that the concentration varies greatly by batch and the ratio of PC to BSA can also vary. This entails the production of a sufficient volume for each study and the dilution of the PC-BSA in coating buffer should be optimised for each batch. During assay

development, several factors were optimised including antibody concentrations and blocking agents. Dilution of the standard pool and samples revealed parallel curves with a plateau at low dilutions.

Assay validation showed that the assay performance was within acceptable limits with inter-assay CV values in the range of 5.75-10.43% and intra-assay CV values in the range of 1.98-11.63%. In general, CV values should be lower than 10% , however in the case of immunoassays, they can be accepted until 20%, particularly if the concentrations of the analyte are low (FDA, 2001). The correlation with this assay and the Tridelta assay was significantly high (r^2=0.84, $p<0.001$), with outliers predominantly in the higher range of CRP concentrations. These would be further diluted in practice.

The advantages of this assay over the previously described assays were the specificity of the antibody to porcine CRP, the ELISA format allowing high throughput of samples, the precision and the low limit of detection. The only disadvantage of this assay is the reliance on species cross-reactivity as this must be checked with each batch of antibody. This could be overcome by the production of a monoclonal antibody to porcine CRP but unfortunately this was beyond the time constraints of this study.

The assay was considered validated for use and was used in the measurement of porcine serum samples in Chapter 3 and Chapter 5 of this thesis.

Chapter III

3. Acute Phase Protein Concentrations in Serum in a High Health Boar Population

3.1.Introduction

A number of studies have shown that APP concentrations in pigs can be indicative of the health status of both individual animals and also the herd as a whole (Petersen *et al.*, 2002a, Burger *et al.*, 1992, Hall *et al.*, 1992, Eckersall, 1987). However, the vast majority of these studies have also shown that APP levels may vary not only due to health status but also age, breed, sex, farm location and husbandry techniques. In order for APP concentrations to be used successfully as health markers, it is first necessary to investigate the baseline concentrations of a herd (measured under typical conditions with no apparent disease) and to ascertain whether the baseline concentrations are influenced by the genetic background of the animal. This allows a 'normal' range of values to be categorised and used as a reference for future measurements.

3.1.1. APP Concentrations in Healthy Pig Populations

As previously mentioned there is a number of porcine APP. In this study, four APP were chosen to be measured. The four selected are among the most widely used and investigated APP and are examples of major (CRP and Hp), pig-specific (Pig-MAP) and negative (TTR) proteins. This approach allowed the use of commercial tests where appropriate and reference material was available for each.

3.1.1.1. Haptoglobin

As previously discussed, normal levels of Hp in pigs are generally <1 g/L but there are age related changes (Martin *et al.*, 2005, Petersen *et al.*, 2002b). Studies have shown that specific pathogen free (SPF) pig herds have a significantly lower Hp serum concentration than conventional herds (Petersen *et al.*, 2002b). Interestingly, there are significant breed differences in Hp serum concentrations with Meishan pigs showing higher Hp concentrations than both the Landrace and Yorkshire breeds (Sutherland *et al.*, 2006). A similar study in commercial pig lines showed a significant difference between two lines, however, this was based on only 18 pigs per line (Frank *et al.*, 2005). In contrast, it has been reported that no differences occur in Hp concentration between Meishan and Large White (LW) breeds (Clapperton *et al.*, 2005b).

3.1.1.2. C-Reactive Protein

Normal concentrations of CRP are reported to be below 100 mg/L but little research has been performed to examine the effects of age or the difference between SPF and conventional herds of pigs on serum concentrations (Pallares *et al.*In press). However, one study has been carried out in which a significant difference was found between the CRP serum concentrations of two different commercial lines (Frank *et al.*, 2005).

3.1.1.3. Pig-MAP

Normal concentrations of Pig-MAP are categorised as being 0.4 – 0.6 g/L and changes in concentrations (a sharp increase followed by a gradual decrease) due to age are exhibited until weaning (Martin *et al.*, 2005, Carpintero *et al.*, 2005). Differences in

Pig-MAP serum concentration between breeds has been reported with Meishan pigs having a significantly higher Pig-MAP concentration than LW (Clapperton *et al.*, 2007).

3.1.1.4. Transthyretin

Very few studies have been carried out in which the baseline concentrations of TTR have been measured. The two most significant studies show a great deal of variance in their normal measurements with reports of a mean of approximately 300 μg/ml (Campbell *et al.*, 2005) and approximately 150 μg/ml (Pineiro *et al.*, 2007b). No studies have been carried out to investigate the effects of breed, sex or farm health status on TTR serum concentrations.

3.1.2. Correlations between APP Concentrations

Several studies have investigated the correlation between APP concentrations in pigs. It has been reported that Pig-MAP and Hp are significantly correlated ($r = 0.57$; $p<0.05$), and it is hypothesised that this may be due to both being induced by IL-6 and having similar kinetics of production (Clapperton *et al.*, 2007, Pineiro *et al.*, 2003). The studies found no correlations between either Hp or Pig-MAP and AGP or TTR.

The aim of this study was to determine the concentrations of four APP (Hp, CRP, Pig-MAP and TTR) in a high health boar population. In addition to the average herd APP concentrations, the difference in APP concentrations across pig lines was also investigated.

3.2.Materials and Methods

3.2.1. Animals

A high health population of 397 boars was made available for this study by Genus, Plc (PIC). The pigs were born between April 2004 and December 2005 and were bled at approximately 7 months of age and again approximately 1 month later. The population comprised 7 pig lines representing current commercial sire and dam line genetics;

- Line A: Landrace ($n = 47$)
- Line B: Large White (LW) ($n = 52$)
- Line C: Duroc ($n = 65$)
- Line D: Synthetic pure line (75% Duroc, 25% LW) ($n = 43$)
- Line E: Synthetic pure line (Hampshire based) ($n = 78$)
- Line F: Synthetic pure line bred for growth ($n = 56$)
- Line G: Synthetic pure line bred for growth (derived from Line F) ($n = 57$)

3.2.2. Experimental Design

The plan for the experimental design is given in Figure 3.1. Pigs were born on 1 of 2 sites (1A and 1B) which consist of breeding, gestation, farrowing and nursery units. All pigs were processed within 24 hr of birth; each pig was weighed, identified with ear tags, a tissue sample taken for DNA and given an iron dextran injection. At weaning (approx 16 days), pigs were transferred to nursery units where they were housed until 70 days of age. At this time pigs were put 'on-test', pigs were screened and the best

pigs grouped by line and transferred to sites 2A/B. At this point, pigs were fed by electronic feeders in order to gauge feed intake. Off-test measurements for the pigs were leg score (front and back), muscle score, weight, teat number, real time ultrasound of the 10th rib areas and feed intake calculated. If selected, pigs were transported to an isolation unit (3) before being transferred to the main unit (4). Animals were culled from these units if performance traits were not of high enough standard and this was assessed on a weekly basis. A few animals were then moved onto the Genesis unit (Site 5).

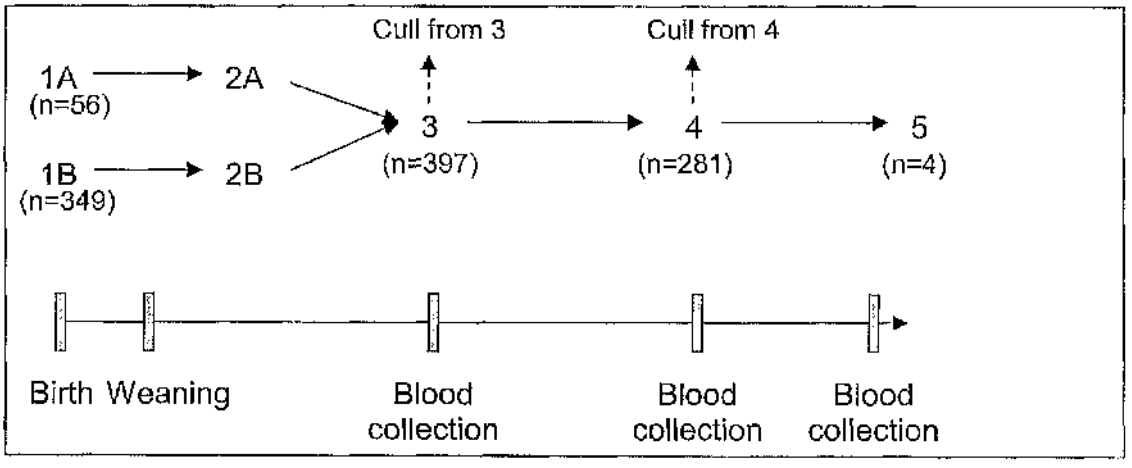


Figure 3.1: Experimental timeline describing movement of pigs through farm sites and blood collection points. 1A: Aurora GN, 1B: Bluegrass (GN), 2A: Wright's isolation, 2B: Mt. Gilead (nursery), 3: KY AI Isolation, 4: KY AI, 5: Genesis. Blood collection was dependent upon age and took place on site 3 at seven months of age and either site 4 or 5, approximately one month later.

3.2.3. Farm Health Status

The farms were all considered to be of a high health status and were porcine reproductive and respiratory syndrome (PRRS) negative, *M. hyopneumoniae* negative and swine influenza virus (SIV) negative. Boars at site 1A (Figure 3.1) were routinely

vaccinated for *H. parasuis*, erysipelas and ileitis and for erysipelas and ileitis at site 1B. Monthly bleeds were carried out by a licensed vet in order to check for notifiable diseases and for certification purposes.

3.2.4. Blood Collection

Blood was obtained by a licensed vet by puncturing the external jugular vein and collecting into anticoagulant (EDTA). Tubes were mixed several times and stored on ice until returned to the laboratory. Upon return, tubes were centrifuged at 800 xg for 10 min and the plasma decanted and frozen at -80°C until shipment on ice to the UK. On arrival in the UK, tubes were stored at -20°C until required for analysis. Throughout this thesis, the samples are referred to as serum samples.

3.2.5. Determination of Acute Phase Protein Concentration

Unless otherwise stated, all chemical reagents were purchased from Sigma-Aldrich, Poole, UK. Double distilled water was used throughout.

3.2.5.1. Haptoglobin

The concentration of Hp in serum was measured using an automated biochemical assay (Eckersall *et al.*, 1999). This assay utilises the innate peroxidase activity of the haemoglobin-haptoglobin (Hb-Hp) complex which can be quantified at low pH. The use of the novel reagent chromogen, (International Patent Application no. PCT/GB98/03407) prevents interference from serum albumin interacting with haem

from lysed cells which can produce false positive results. A working solution of haemoglobin (Hb) was prepared by adding 56 µl of a stock solution of equine Hb (30 g/L) to 25 ml 0.9% (w/v) NaCl. The substrate was prepared by the addition of 100 µl of H_2O_2 to 25 ml H_2O. The reagent used was a stock solution of chromogen buffer (courtesy of Mary Waterston, Faculty of Veterinary Medicine, University of Glasgow). Standards were prepared using bovine acute phase serum with a Hp concentration of 1.48 g/L (calibrated against the European Standard for the porcine acute phase proteins: European Concerted Action QLK5-CT-1999-0153) and diluted to 0.73 g/L, 0.38 g/L with 2% BSA and using the 2% BSA as a zero. Control samples of serum with known Hp concentrations, 0.9% NaCl and 2% BSA were run in each assay.

The following protocol for the measurement of Hp on the Cobas MIRA analyser (Roche Diagnostics Ltd., Switzerland) was used. Serum (3.5 µl sample or standard) was mixed with 100 µl Hb in the reaction cuvette. After 50 sec, 45 µl chromogen reagent was added, and after a further 25 sec, 25 µl substrate was added. The absorbance at 600 nm was measured and the difference in absorption over the next 50 sec was used to calculate a standard curve. The change in absorbance of the samples was compared to the standard curve and the results were reported as g/L.

3.2.5.2. C-Reactive Protein

The concentration of CRP in serum was determined using the ligand-binding assay developed in Chapter 2. Internal controls were run in each assay.

3.2.5.3. Pig-MAP

A commercial ELISA (PigMAP Kit ELISA, PigCHAMP Pro Europa, Segovia, Spain) was used for the measurement of Pig-MAP in serum. This is a sandwich ELISA based upon 2 monoclonal antibodies and has been calibrated against a standard of known Pig-MAP concentration, according to the European Standard for the porcine acute phase proteins.

The assay was used according to the manufacturer's protocol with all steps carried out at room temperature (RT). Briefly, samples and internal controls were applied at a 1:1000 dilution in assay buffer to antibody coated microwells and incubated for 30 min. After washing 4 times with buffer the peroxidase conjugated antibody was applied and incubated for 30 min before washing again. Chromogen substrate solution was added and 30 min later, stop solution added, the final absorbance was read at 450 nm using the FLUOstar OPTIMA platereader and the results calculated by the associated FLUOstar Optima Software V1.32.

3.2.5.4. Transthyretin

A modified in-house ELISA technique was followed to determine the concentration of TTR in serum (Campbell *et al.*, 2005). Microtitreplates (96 well, Nunc) were coated with 100 µl/well of either serum samples or internal controls (diluted 1:2000 with 50 mM $NaHCO_3$, pH 9.6) or purified human prealbumin (TTR) (Sigma) at concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.0312 µg/ml (diluted in 50mM $NaHCO_3$, pH 9.6). The plates were then incubated overnight at 4°C. Following incubation, the samples

were decanted and any unbound sites blocked with 250 µl 5% (w/v) non-fat milk in PBS-Tween (0.12 M NaCl, 0.02 M Na_2HPO_4, pH 7.4, 0.1% (v/v) Tween 20) for 30 min at room temperature. The plates were then washed three times with PBS-Tween before 100 µl of sheep polyclonal to prealbumin (sheep anti-human TTR, Abcam, Cambridge, UK) (diluted 1:5000 with PBS-Tween) was added to each well and the plates incubated for 1 hr at 37°C. Following incubation the plates were washed three times in PBS-Tween then 100 µl of HRP conjugated donkey anti-sheep IgG (Sigma) (diluted 1:5000 in PBS-Tween) was added to each well and the plates incubated at 37°C for 30 min. Following three washes in PBS-Tween, 100 µl of fresh TMB substrate solution (KPL) was added to each well and the reaction stopped after 7 to 10 minutes with 50 µl of 1 M HCl. The absorbance was read at 450 nm using the FLUOstar OPTIMA platereader (BMG Labtech Ltd.) and the results calculated by the associated FLUOstar Optima Software V1.32.

3.2.6. Data Analysis

Statistical analysis was carried out using SAS 9.1 software (The SAS Institute, Cary, NC, USA). All data was log transformed prior to analysis in order to render skewed data as close to normal distributions as possible. Correlations between the APP were analysed using the PROC CORR function of SAS 9.1. Associations between pig line data were analysed using the General Linear Model (GLM) procedure of SAS 9.1.

3.3.Results

3.3.1. Precision of APP Tests

The intra and inter-assay coefficients of variation (CVs) are shown in Table 3.1. Intra-assay CVs for the CRP ELISA have already been reported in Section 2.3.5.1. Pig-MAP was measured using a commercial assay which has previously been validated for use (Teeles *et al.*, 2007), and as such, intra-assay CVs were not calculated for this assay.

3.3.2. Correlation between APP Concentrations from Repeated Sampling

Two bleeds were taken from most pigs. Statistical analysis showed that the 2 bleeds were significantly correlated in all 4 of the proteins; Hp, $r = 0.301$ ($p < 0.0001$, n=397), CRP, $r = 0.269$ ($p < 0.0001$, n=395), Pig-MAP, $r = 0.210$ ($p = 0.002$, n=396) and TTR, $r = 0.599$ ($p < 0.0001$, n=397). This enabled the average of the 2 bleeds to be used for further analysis.

3.3.3. Serum Concentrations of APP in a High Health Herd

Serum concentrations of Hp, CRP, Pig-MAP and TTR across a high health herd are shown in Figures 3.2 – 3.5 respectively. The mean Hp, CRP and Pig-MAP concentrations were 0.4 g/L (range = 0 – 2.8 g/L), 29.85 mg/L (range = 0.9 – 485.2 mg/L) and 1.2 mg/ml (range = 0.1 – 9.6 mg/ml), respectively. The mean TTR concentration was 337.6 μg/ml (range = 119.2 – 820.3 μg/ml). Summary values are shown in Table 3.2.

Table 3.1: Intra- and inter-assay coefficients of variation (CVs) for Hp, CRP, Pig-MAP and TTR assays.

	Intra-assay			Inter-assay		
	n	Mean (SD)	CV %	n	Mean (SD)	CV %
Hp (g/L)	6	0.37 (0.02)	5.08	26	0.29 (0.06)	20.06
	6	0.65 (0.03)	4.07	26	0.70 (0.11)	16.24
	6	0.92 (0.05)	4.91	26	1.03 (0.15)	14.41
CRP (mg/L)	-	-	-	21	85.8 (12.1)	14.13
	-	-	-	21	182.1 (31.1)	12.96
	-	-	-	21	276.3 (35.8)	17.08
Pig-MAP (mg/ml)	-	-	-	22	0.7 (0.3)	42.63
	-	-	-	22	2.0 (0.5)	22.83
TTR (µg/ml)	6	148.6 (7.7)	5.16	31	144.01 (30.4)	21.13
	6	285.8 (6.9)	2.40	31	275.6 (73.9)	26.82
	6	312.0 (6.7)	2.15	31	366.5 (113.9)	31.07

Intra-assay CVs for CRP have been previously reported. Pig-MAP has previously been validated for use.

Table 3.2: Summary values of Hp, CRP, Pig-MAP and TTR concentration in a high health herd.

	Hp (g/L)	CRP (mg/L)	Pig-MAP (mg/ml)	TTR (µg/ml)
n	397	395	396	397
Min.	0.0	0.9	0.1	119.2
Max.	2.8	485.2	9.6	820.3
Median	0.3	13.5	1.1	323.8
Mean	0.4	29.8	1.2	337.6
SEM	0.02	2.7	0.04	5.53
SD	0.37	54.49	0.78	110.26

Abbreviations: *n*: no. of pigs. SEM: standard error of the mean. SD: standard deviation

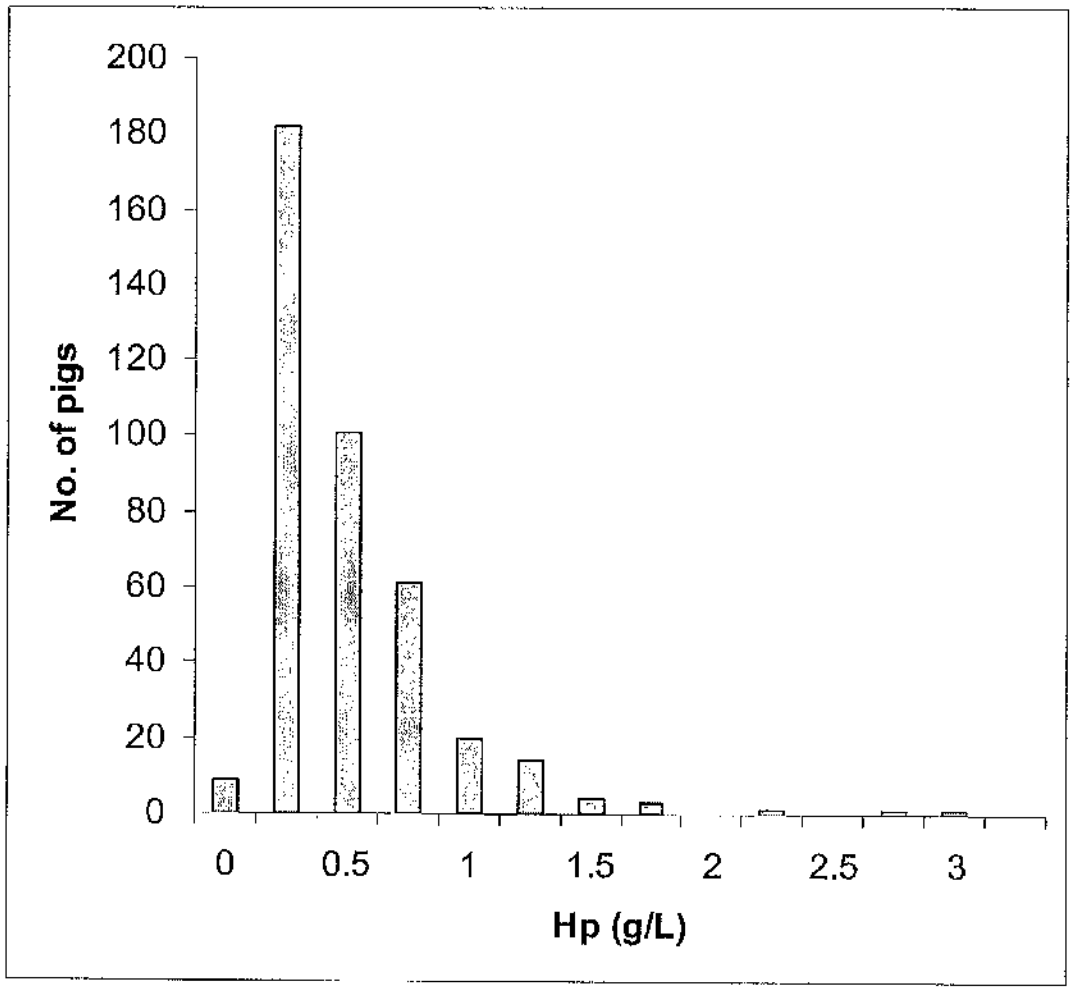


Figure 3.2: Serum concentrations of Hp (g/L) in a high health pig herd (n = 397).

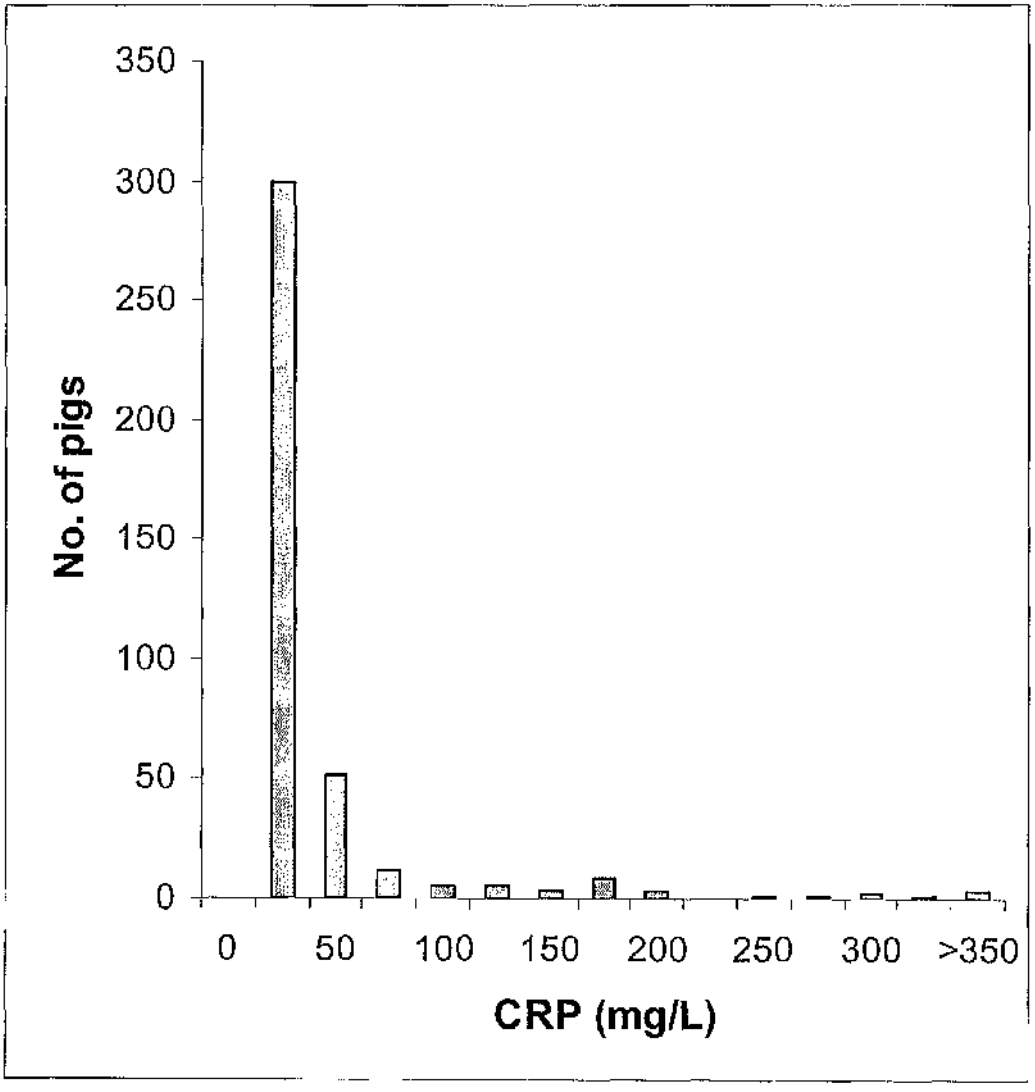


Figure 3.3: Serum concentrations of CRP (mg/L) in a high health pig herd (n = 395).

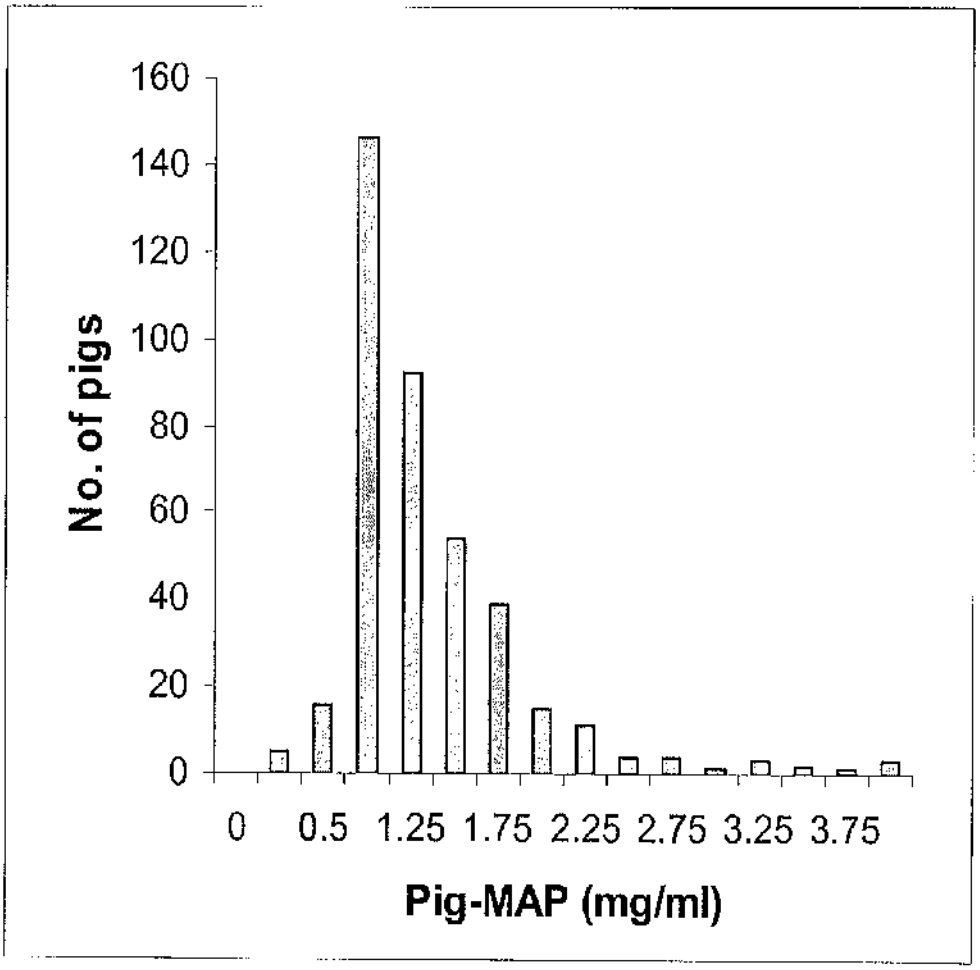


Figure 3.4: Serum concentrations of Pig-MAP (mg/ml) in a high health pig herd (n = 396).

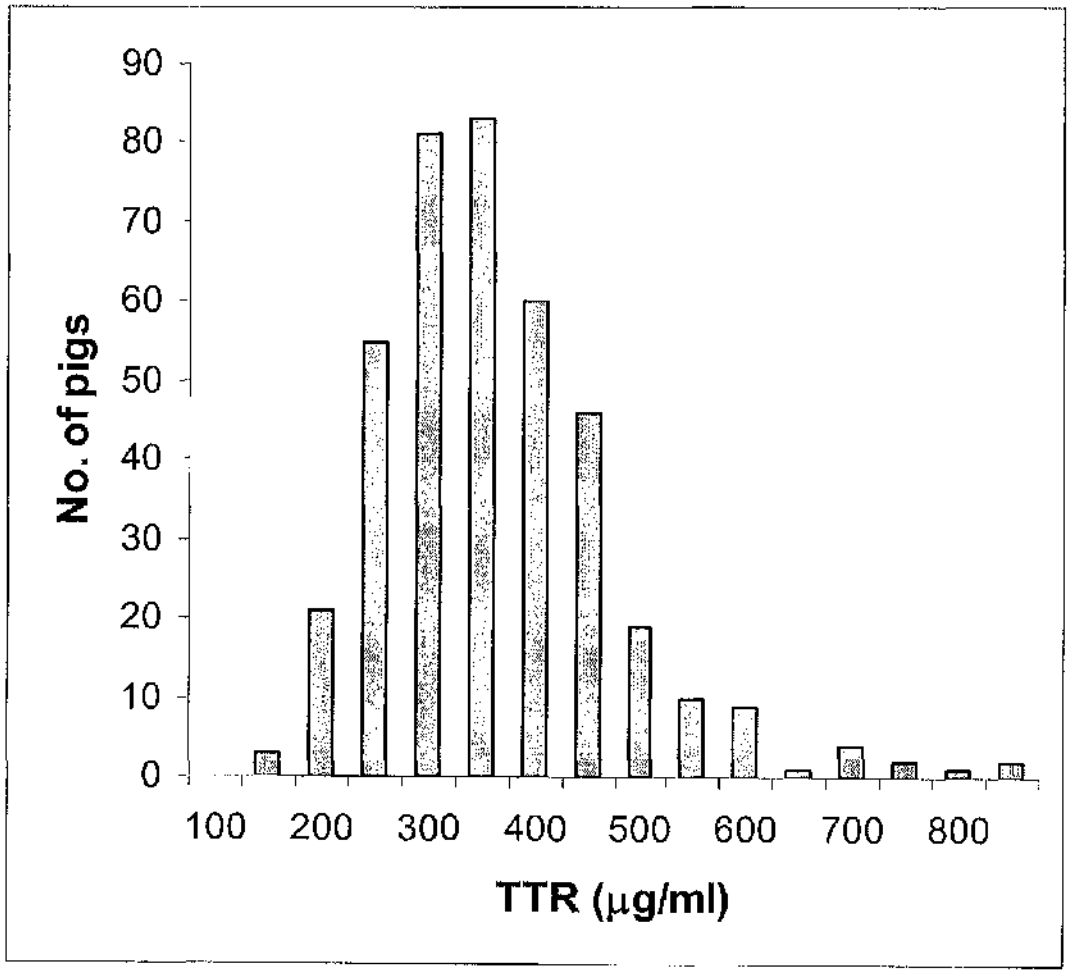


Figure 3.5: Serum concentrations of TTR (µg/ml) in a high health pig herd (n = 397).

3.3.4. Correlations between APP Concentrations

Correlations between the 4 measured APP are shown in Table 3.3. There was a significant correlation between Hp and CRP ($p < 0.0001$), between Hp and Pig-MAP ($p < 0.0001$) and between CRP and Pig-MAP ($p < 0.0001$) (see also Figures 3.6 - 3.8). There was no correlation found between TTR and any of the other 3 proteins.

Table 3.3: Correlation coefficients (r) describing the relationship between Hp, CRP, Pig-MAP and TTR.

	Hp	CRP	Pig-MAP	TTR
Hp	.	0.537*	0.295*	0.064
CRP	0.537*	.	0.336*	0.040
Pig-MAP	0.295*	0.336*	.	0.105
TTR	0.064	0.040	0.064	.

* $p < 0.0001$

Note: Analysis carried out using log-transformed data.

3.3.5. Comparison of Serum Concentrations of APP across Pig Lines

Serum concentrations of Hp, CRP, Pig-MAP and TTR in all lines are shown in Figures 3.9 - 3.12 respectively. Summary values are given in Tables 3.4 - 3.7. There was no significant difference in Hp concentration between pig lines. There was a significant difference found between lines for CRP ($p = 0.0121$), Pig-MAP ($p = 0.0426$) and TTR ($p < 0.0001$).

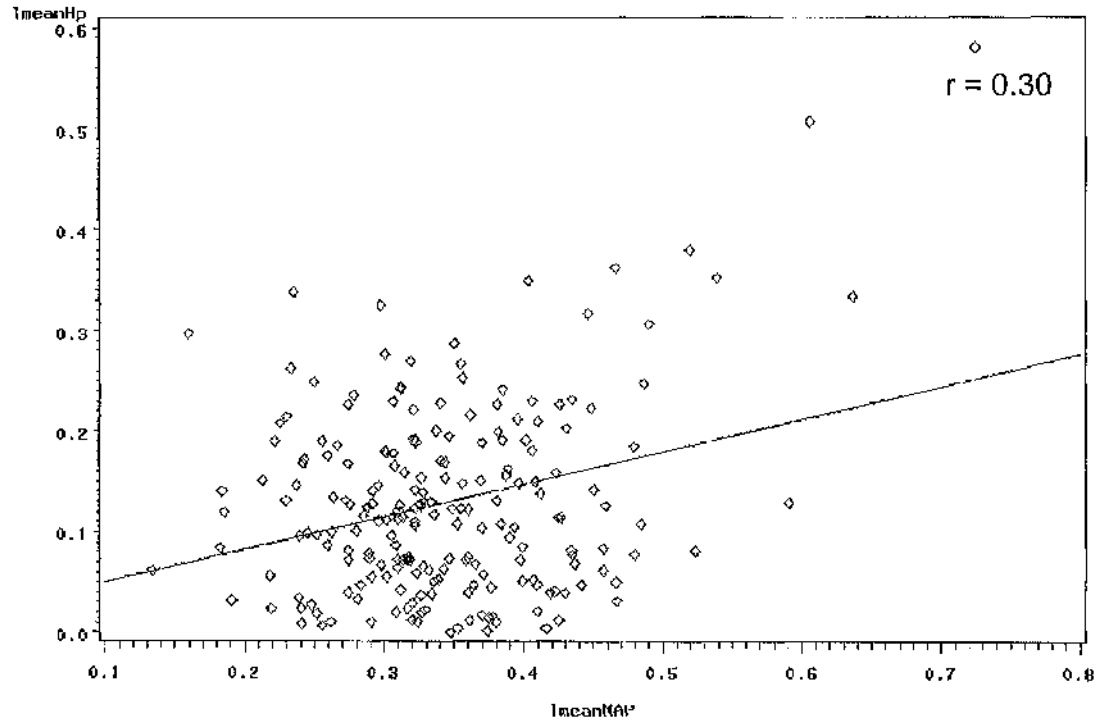


Figure 3.6: Plot of Pig-MAP vs. Hp. Data was log transformed before analysis. **Abbreviations:** lmeanHp, log transformed haptoglobin; lmeanMAP, log-transformed Pig-MAP.

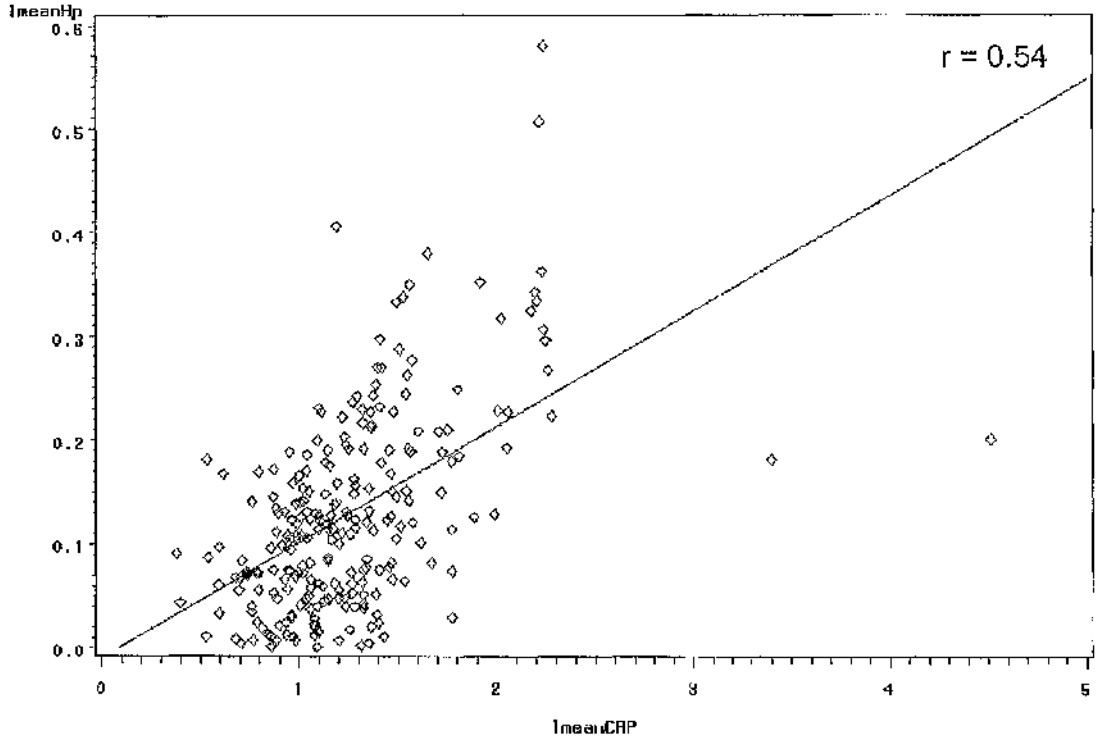


Figure 3.7: Plot of Hp vs. CRP. Data was log transformed before analysis. **Abbreviations:** lmeanHp, log-transformed Haptoglobin; lmeanCRP, log-transformed C-reactive-protein.

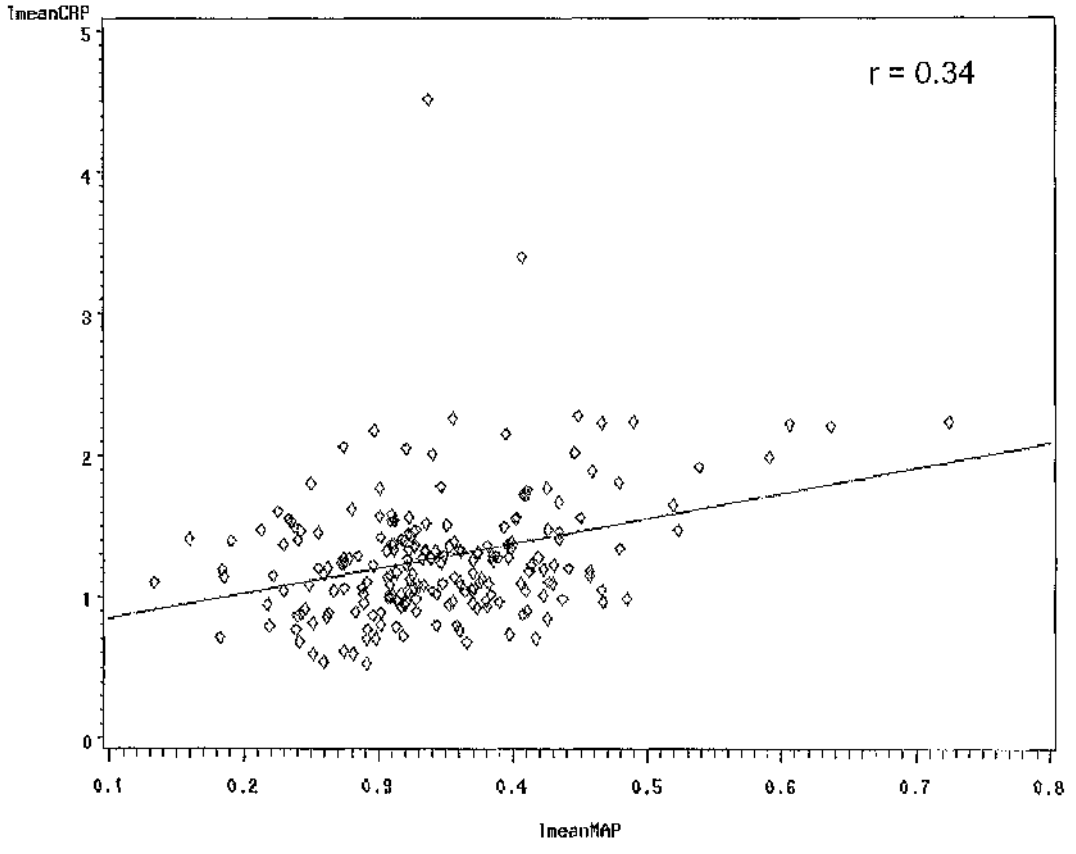


Figure 3.8: Plot of CRP vs. Pig-MAP. Data was log transformed before analysis. **Abbreviations:** lmeanCRP, log-transformed C-reactive protein; lmeanMAP, log-transformed Pig-MAP.

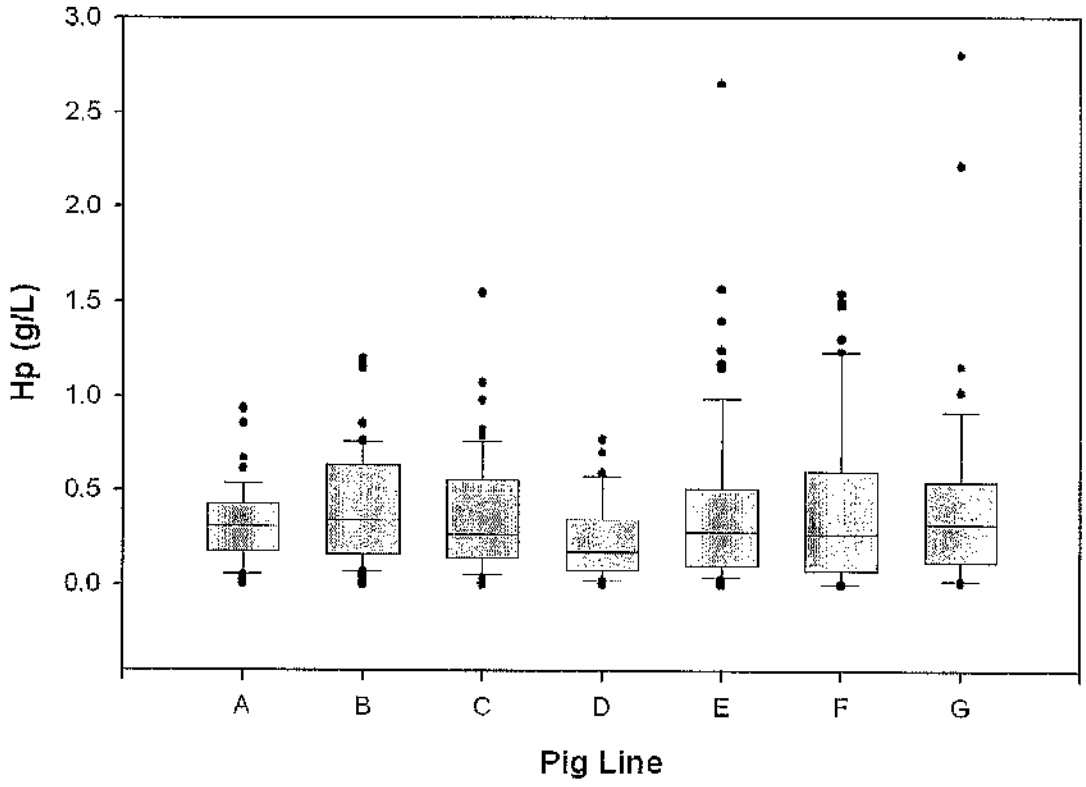


Figure 3.9: Hp concentration (g/L) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

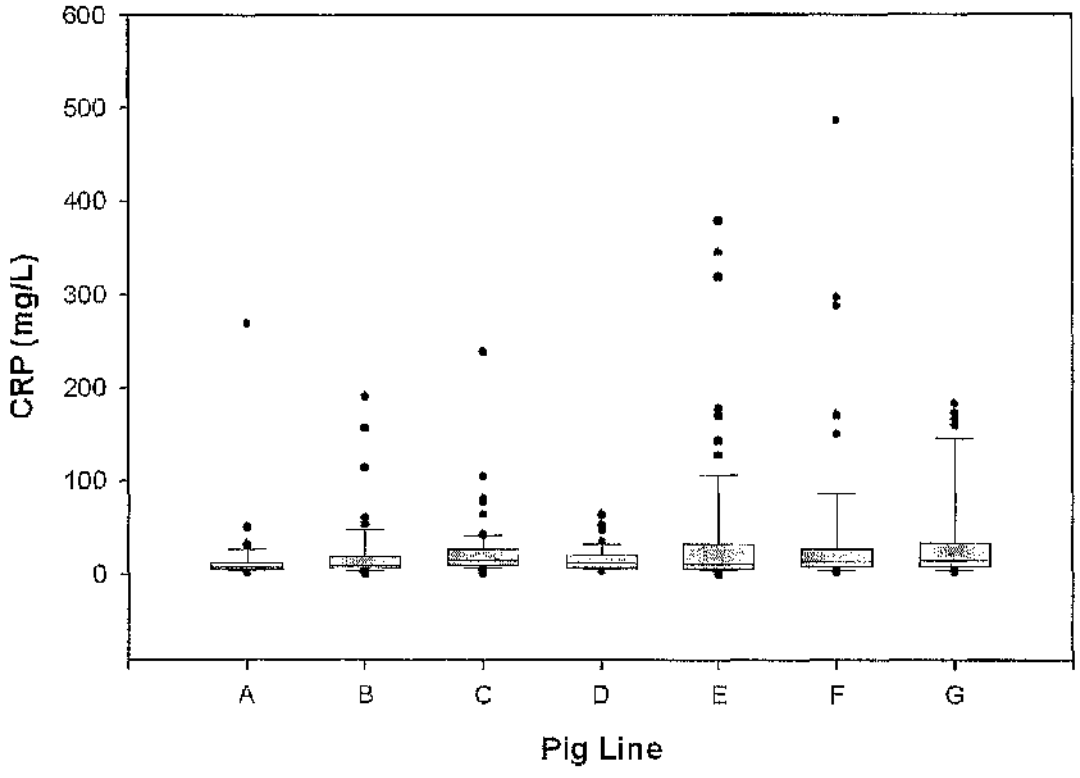


Figure 3.10: CRP concentration (mg/L) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

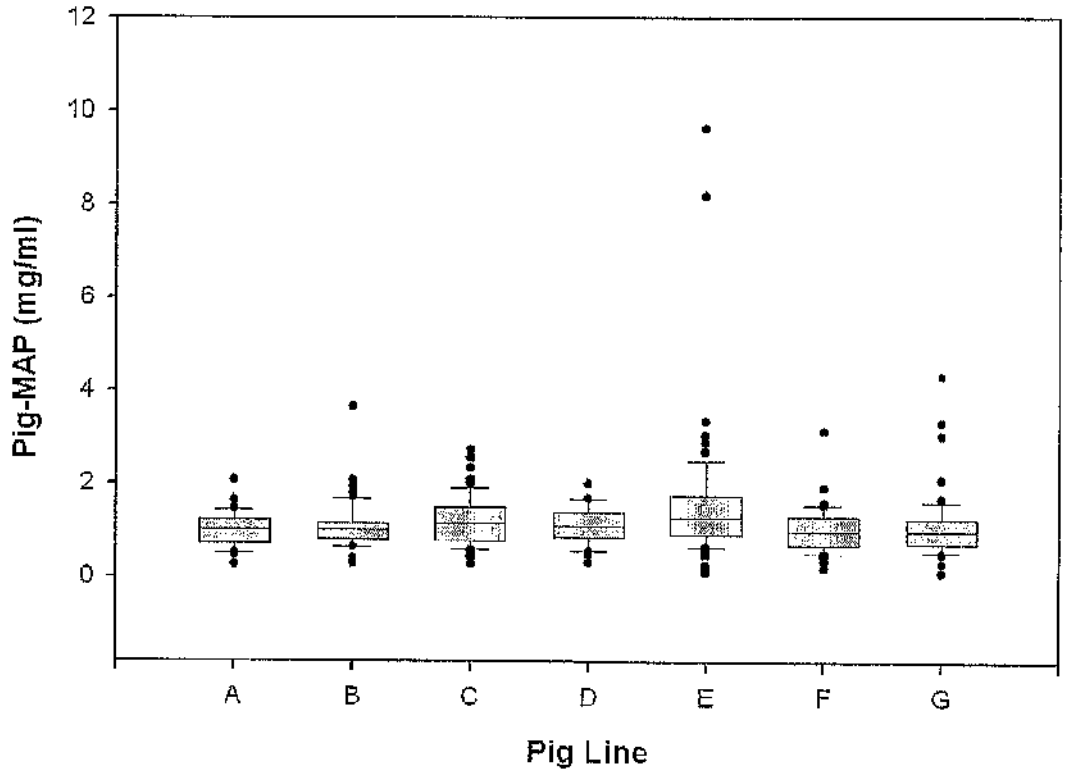


Figure 3.11: Pig-MAP concentration (mg/ml) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

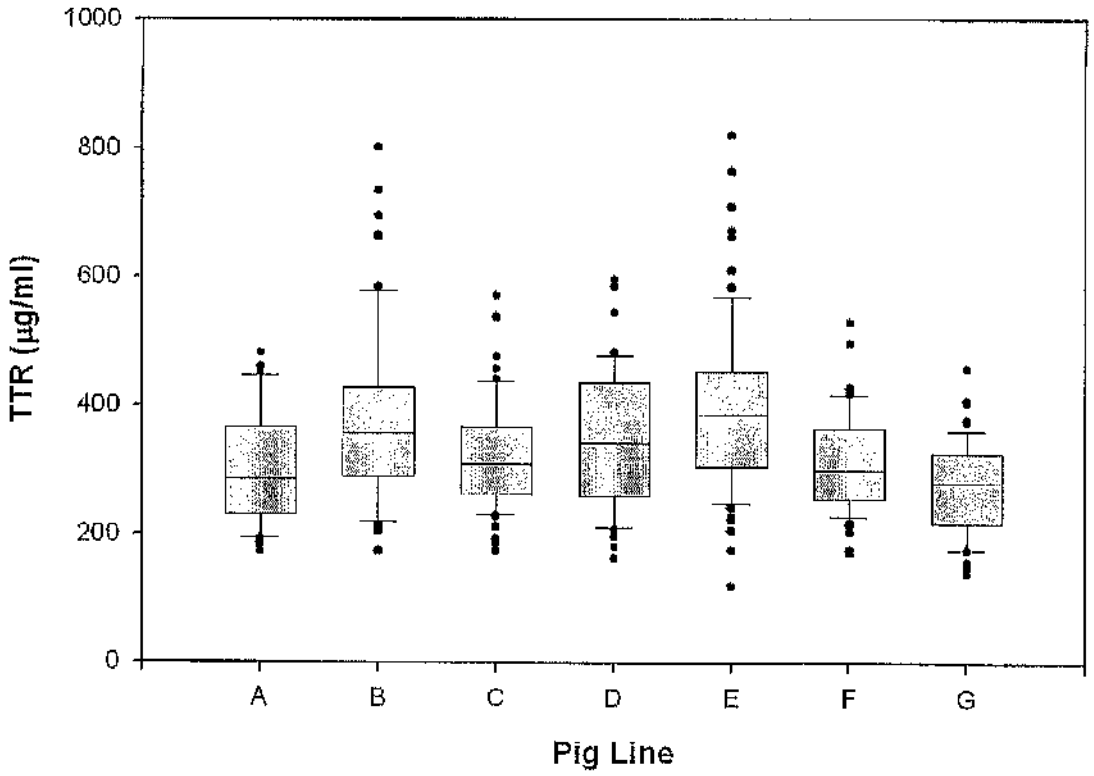


Figure 3.12: TTR concentration (µg/ml) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

Table 3.4: Summary values of Hp (g/L) across 7 pig lines (n = 397).

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	46	52	65	43	78	56	57
Min.	0.01	0.00	0.00	0.00	0.00	0.00	0.02
Max	0.94	1.20	1.55	0.77	2.65	1.54	2.80
Median	0.31	0.35	0.27	0.18	0.29	0.27	0.32
Mean	0.31	0.42	0.36	0.23	0.40	0.40	0.43
SEM	0.03	0.04	0.04	0.03	0.05	0.06	0.07
SD	0.21	0.31	0.30	0.20	0.44	0.43	0.49

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation

Table 3.5: Summary values of CRP (mg/L) across 7 pig lines (n = 395).

	Line A[a]	Line B[b]	Line C[a]	Line D	Line E	Line F[a]	Line G[ab]
n	47	52	65	43	76	56	57
Min.	3.6	2.4	2.4	3.9	0.9	3.4	3.6
Max	269.5	191.1	238.7	63.9	378.1	485.2	183.0
Median	8.8	11.0	15.9	13.5	12.6	14.6	16.0
Mean	17.4	22.9	25.2	17.5	38.5	41.3	38.0
SEM	5.76	4.93	4.06	1.95	8.32	11.24	6.71
SD	39.06	35.57	32.70	12.76	72.51	84.09	50.67

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation
The same superscript indicates a significant difference ($p<0.05$) in serum concentrations between the marked lines.

Table 3.6: Summary values of Pig-MAP (mg/ml) across 7 pig lines ($n = 396$).

	Line A[a]	Line B[b]	Line C	Line D	Line E[abcd]	Line F[c]	Line G[d]
n	46	52	65	43	77	56	57
Min.	0.28	0.29	0.29	0.34	0.11	0.21	0.13
Max	2.07	3.64	2.45	2.02	9.63	3.13	4.31
Median	1.03	1.03	1.15	1.12	1.30	1.02	1.00
Mean	1.01	1.09	1.22	1.14	1.57	1.04	1.12
SEM	0.05	0.07	0.07	0.06	0.16	0.06	0.09
SD	0.35	0.51	0.53	0.38	1.38	0.47	0.69

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation.
The same superscript indicates a significant difference ($p<0.05$) in serum concentrations between the marked lines.

Table 3.7: Summary values of TTR (μg/ml) across 7 pig lines ($n = 397$).

	Line A[abc]	Line B[ad]	Line C[ef]	Line D[bg]	Line E[cehi]	Line F[hj]	Line G[dfgij]
n	46	52	65	43	78	56	57
Min.	172.0	173.4	173.6	162.0	119.2	170.7	138.3
Max	482.0	801.8	571.4	595.9	820.3	529.3	457.8
Median	286.0	357.8	310.0	341.6	385.8	300.9	281.3
Mean	303.6	379.6	324.9	346.4	396.4	314.9	276.5
SEM	12.58	19.05	10.44	16.52	14.78	10.11	9.39
SD	85.35	137.35	84.20	108.36	130.50	75.65	70.88

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation
The same superscript indicates a significant difference ($p<0.05$) in serum concentrations between the marked lines.

3.4.Discussion

The aim of this study was to examine serum concentrations of four APP (Hp, CRP, Pig-MAP and TTR) in a high health boar population. The study included determining average 'herd' baseline concentrations, identifying correlations between the APP and determining if any differences in serum concentration occurred between pig lines.

Baseline APP serum concentrations were determined using a variety of assays (3 'in-house' and 1 commercial assay). The assays were validated for use by running internal controls and determining intra- and inter-assay CVs. Intra-assay CVs were below 10% which is optimal for analytical determinations. Inter-assay CVs were within the range of 12 – 43%, with the highest CV value occurring for an internal control in a commercial assay (pig-MAP). Values of up to approximately 30% are acceptable for ELISA (this accounts for the CRP and TTR assays) and were deemed acceptable for Hp, particularly towards the lower and upper limits of detection and it was deemed not necessary to repeat these assays (FDA, 2001). The Pig-MAP ELISA has been previously validated for use by Tecles *et al.* (2006) in which it also showed a high CV of 23.1%. Taking this into account, also with the fact that only the internal control of low Pig-MAP concentration at 0.66 g/ L showed a CV of 43%, and the cost (both time and monetary), it was decided not to repeat the Pig-MAP assays (Tecles *et al.*, 2007).

This study used boars from a high health herd with similar housing and husbandry regimes and had blood collected at approximately the same age which was repeated a month later. The APP serum concentrations from the two bleeds were shown to be significantly correlated and therefore it was possible in the statistical analysis to use the average of both the bleeds. By taking this approach, and minimising treatment

differences between the boars, the concentration of serum APP could be considered to be an average baseline level and any differences in concentration should therefore theoretically be due to genetic differences between the animals.

Across the boar population, the average baseline concentrations of Hp, CRP and TTR were within the reported ranges for healthy animals (Campbell *et al.*, 2005, Petersen *et al.*, 2002b, Heegaard *et al.*, 1998) and showed a distinct trend towards the lower range of Hp and CRP concentration and the higher range of TTR concentration. In all three cases, there were very few boar samples with APP concentrations outside the mean concentration ± 2SD; this indicates that there was no sub-clinical disease or underlying problems, as would be expected in a high health herd. In the case of Pig-MAP, baseline concentrations have previously been categorised as between 0.4 and 0.6 mg/ml after weaning (Carpintero *et al.*, 2005, Martin *et al.*, 2005). This study showed an average baseline Pig-MAP concentration of 1.19 mg/ml, almost twice the previous reports. This might have sparked concern if Pig-MAP had been the only protein measured, as it would indicate a possible health and welfare issue. However, a recent study also reported baseline concentrations closer to 1 mg/ml (Clapperton *et al.*, 2007). The difference between these values could be due to a number of factors such as age, sex and breed but it does highlight two important issues; first that it is necessary for baseline APP concentrations to be adjusted for factors such as sex and/or be determined for individual farms or herds. Secondly, measurement of at least two APP is recommended in order to gain a better understanding of what biological processes are occurring.

The study of baseline concentrations of the porcine APP has also raised questions over the classification of the APP as major or moderate. Historically, pCRP has always been

considered a major APP due to its dramatic and almost immediate increase (approximately 10-15 times) in concentration during an APR (Parra *et al.*, 2006, Lampreave *et al.*, 1994). However, the baseline concentration of pCRP is considered high for a major APP, particularly when compared to the baseline concentrations of human (~1 mg/L) (Danesh *et al.*, 2004, Pepys and Hirschfield, 2003) or dog (<10 mg/L) (Ceron *et al.*, 2005) . This had led to questions over its categorisation as a major APP and is an area which should be further investigated.

Analysis of the herd APP concentrations showed a significant correlation between Hp and CRP, Hp and Pig-MAP and Pig-MAP and CRP. The correlation between Hp and Pig-MAP has previously been reported, however no studies had been carried out investigating the relationship with CRP. All 3 proteins are stimulated by IL-6 (Gonzalez-Ramon *et al.*, 2000, Kushner and Rzewnicki, 1994), and it has been hypothesised that this may be responsible for the correlation between them (Clapperton *et al.*, 2007). It is worth noting that in this study, the highest correlation was found between CRP and Hp both of which are considered major APP, with lower correlations found between either of these and Pig-MAP, a moderate APP. This suggests that correlation between APP is affected by multiple factors and not just the mediator stimulating their synthesis. This is further corroborated by the lack of correlation with transthyretin, a negative APP. The correlation between baseline concentrations of Hp, CRP and Pig-MAP could be put to a practical use as it means that only two APP need to be measured. These could be chosen on the grounds of cost, speed or availability of assays, or it could be considered that measurement of one major and one moderate APP is more appropriate.

The concentration of APP was compared between 7 lines comprising pure European breed lines and synthetic lines. Studies have indicated that there are significant breed and/or line differences in CRP and Pig-MAP particularly between Asian and European breeds (Clapperton *et al.*, 2007, Frank *et al.*, 2005). There are conflicting reports concerning Hp, with one study indicating that differences occur between Asian and European breeds (Sutherland *et al.*, 2006) and one study finding no difference (Clapperton *et al.*, 2005b). This study found that there were no line differences for Hp serum concentration supporting the latter study (neither of these studies included Asian breeds). Differences between lines were discovered for CRP, Pig-MAP and TTR serum concentrations. In particular, differences in CRP concentration were found between Line A (Landrace) and Lines C (Duroc), F and G (both synthetic pure lines bred for growth), with Line A showing a lower mean CRP concentration than the others. Differences in Pig-MAP concentration occurred between Line E (Synthetic pure line based on Hampshire) and Lines A, B (LW), F and G, with Line E showing a higher mean Pig-MAP concentrations than the other lines. Differences in TTR concentration occurred between almost every line (Line E>Line B>Line D>Line C>Line F>Line G) but of particular interest were Line F and G. Line G is derived from Line F, yet they have significantly different TTR concentrations, this may be an effect of selecting for a particular trait.

The differences in APP concentrations between lines are likely to be genetic and could be attributed to polymorphisms in the genes controlling APP production; either the APP genes themselves or possibly the genes involved in control mechanisms such as the cytokine genes. However, there is obviously a clear difference between the lines and this has also been shown for other immune traits (Clapperton *et al.*, 2005b, Sutherland

et al., 2005). As yet, the underlying cause and purpose of this difference is unknown. This study also shows the effect that differential selection could have on an immune trait, as exhibited in Lines F and H; however, elucidation of this outcome would require further investigation.

This study has determined the average baseline APP serum concentrations for a high health boar population (as determined by industry standards) and identified line differences in baseline CRP and TTR serum concentrations that have not been previously reported. It also provides further evidence for line differences in Pig-MAP serum concentration. In addition, overall correlations between CRP and Hp and Pig-MAP concentrations have been identified and a previously reported correlation between Hp and Pig-MAP corroborated. This data adds to our knowledge of APP biology in pigs, particularly the effect of selective breeding and has identified areas requiring further investigation such as identification of factors affecting APP baseline concentrations i.e. line and the effects of selective breeding on immune traits such as the APP.

Chapter IV

4. Identification of Single Nucleotide Polymorphisms in Acute Phase Protein Genes

4.1.Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNP) involve the substitution of one nucleotide for another and are normally bi-allelic but can have three or even four alleles. Most SNP are located in non-coding regions of the genome and although it is thought they may contribute to gene expression, they usually have no direct impact on the phenotype of an individual. However, these non-coding SNP can be in high or complete linkage disequilibrium (LD) with causative mutations and these can still be used as markers. Single nucleotide polymorphisms occurring in the coding regions of genes can alter the structure or function of the encoded proteins and are the cause of most of the known inherited monogenic disorders (Syvanen, 2001, Landegren *et al.*, 1998). There are four major reasons for the interest in SNP as genetic markers. Firstly, they are more prevalent in the genome than other types of polymorphisms, occurring approximately every 1000 base pair (bp) in humans (Landegren *et al.*, 1998); approximately every 500 bp in mice and cattle (Lindblad-Toh *et al.*, 2000, Heaton *et al.*, 2001) and approximately every 400 bp in pigs (Jungerius *et al.*, 2003). In addition, they generally give rise to large sets of markers lying near or in the locus of interest. Secondly, some SNP in genes can change the amino acid sequence of a protein (known as non-synonymous SNP), directly affecting protein structure or expression levels and can be considered candidate alterations for genetic mechanisms in disease.

Thirdly, they are stably inherited, making them ideal for long-term selection programmes and finally, typing systems for SNP can offer high throughput and efficiency, leading to large power in genetic analyses (Beuzen *et al.*, 2000, Landegren *et al.*, 1998).

4.1.1. Single Nucleotide Discovery and Genotyping

The simplest and most common method of SNP discovery is to target an area of interest in the genome, for example one containing candidate genes. Regions of the genes can then be sequenced following PCR amplification of either individual or pooled DNA and SNP identified manually or by the use of computer software (Vignal *et al.*, 2002).

4.1.2. Use of BAC End Sequencing

Bacterial artificial chromosomes (BAC) are common vectors used for cloning up to 300 kb of genomic sequence. They are a valuable resource for whole genome and targeted genome sequencing and are currently being used in the porcine genome sequencing project (http://www.sanger.ac.uk/Projects/S_scrofa/mapping.shtml). BAC end sequences (BES) can be mapped against the human genome and sequences selected that lie in the region of human genome in which the genes of interest lie. This approach has been used in human, mouse and cattle projects enabling detailed physical maps to be made. Additionally, BES can be used to generate oligonucleotides to be used for amplifying and sequencing genomic DNA (Fahrenkrug *et al.*, 2001, Suzuki *et al.*, 2000). Several porcine genomic BAC

libraries are available (Anderson *et al.*, 2000, Suzuki *et al.*, 2000, Rogel-Gaillard *et al.*, 1999).

4.1.3. Genotyping

Once SNP have been identified there are a large number of techniques available for SNP genotyping as indicated in Table 4.1, each with its own advantages and disadvantages. Three specific techniques were used in this study and will be discussed in greater detail.

Table 4.1: Single Nucleotide Polymorphism (SNP) Genotyping Methods

Method	Advantages	Disadvantages	Features
PCR-RFLP	Low cost Simple	Qualitative visual interpretation Not all SNP are part of a restriction site	Traditional method
MALDI-TOF MS	High throughput High sensitivity Multi-plexing capacity	Cost Susceptible to contamination	
Gene-Chip Microarrays	High probe density	Cost High failure rate	High tech manufacturing process
SNaPshot®	Multi-plexing capacity	Size separation step	Can be performed on capillary DNA sequencers
Pyrosequencing®	High throughput Sensitive	Difficult to multi-plex Cost	Dedicated instrument
TaqMan®	Simple	Cost	Quantitative real-time PCR
FRET primers	Simple	Cost	Universal FRET primers
Coded microspheres	Multiplexing capacity	Microspheres not widely availalble	Flow cytometric detection

Abbreviations: PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; MALDI-TOF MS: matrix-assisted laser desorption ionisation time-of-flight mass spectrometry; FRET: fluorescence resonance energy transfer. (Isler *et al.*, 2007, Landegren *et al.*, 1998, Syvanen, 2001)

4.1.3.1. PCR-RFLP

PCR-restriction fragment length polymorphism (PCR-RFLP) was one of the first genotyping techniques to be used. It is a simple and powerful technique; however it can be time-consuming and inconvenient. Following amplification of the DNA region of interest, PCR-RFLP uses the ability of restriction enzymes to cleave the DNA sequence at particular sites. If a SNP creates or removes a restriction endonuclease recognition site, then the DNA sequence acquires or loses the ability to be cleaved at that particular recognition site. If no site is present, then enzyme digestion will result in a long fragment, if a recognition site is present, then digestion will results in two shorter fragments. If a recognition site is present in only one allele (for example, a heterozygous individual), digestion will result in one long and two shorter fragments (Beuzen *et al.*, 2000). The main disadvantage to this technique is that some polymorphisms do not result in the creation or disruption of restriction endonuclease sites, leading to the use of alternative techniques.

4.1.3.2. Pyrosequencing®

Pyrosequencing® (Biotage, Uppsala, Sweden) is a relatively new technique, first described in 1998 (Ronaghi *et al.*, 1998), and provides genotyping results in context of the neighbouring DNA sequence. It is a high throughput (96 samples in 5-7 min after PCR and clean-up steps), accurate and low cost technique, and it is seen by many as the future for genotyping (Pati *et al.*, 2004, Ahmadian *et al.*, 2000, Ronaghi *et al.*, 1998).

The Pyrosequencing® technique is based on the detection of released pyrophosphate (PPi) during DNA synthesis and is summarised in Figure 4.1. Following PCR amplification of the target DNA sequence, the sequencing primer is hybridised to a single stranded template isolated from the PCR reaction using streptavidin beads to capture the biotinylated template and incubated with the enzymes; DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates adenosine 5'phosphosulfate (APS) and luciferin (Step 1). Four enzymatic reactions then follow. These start with the addition of one of four deoxyribonucleotide triphosphates (dNTPs) with DNA polymerase to catalyse the incorporation of the dNTP complementary to the base present in the DNA template. The incorporation is accompanied by a release of PPi in proportion to the amount of dNTP incorporated (Step 2). ATP sulfurylase then converts PPi into ATP in the presence of APS; this drives luciferase-mediated conversion of luciferin to oxyluciferin and generates visible light in amounts proportional to the amount of ATP. The light emitted is detected by a camera and is visualised as a peak in a pyrogram™, with the height proportional to the number of nucleotides incorporated (Steps 4 and 5). Apyrase is added to degrade unincorporated dNTPs and excess ATP prior to the addition of the next dNTP (Step 4). Addition of the dNTPs is performed one at a time. As this continues, this allows the complementary DNA strand to be synthesised and the sequence to be determined (Isler *et al.*, 2007, Ronaghi, 2003, Ronaghi *et al.*, 1998).

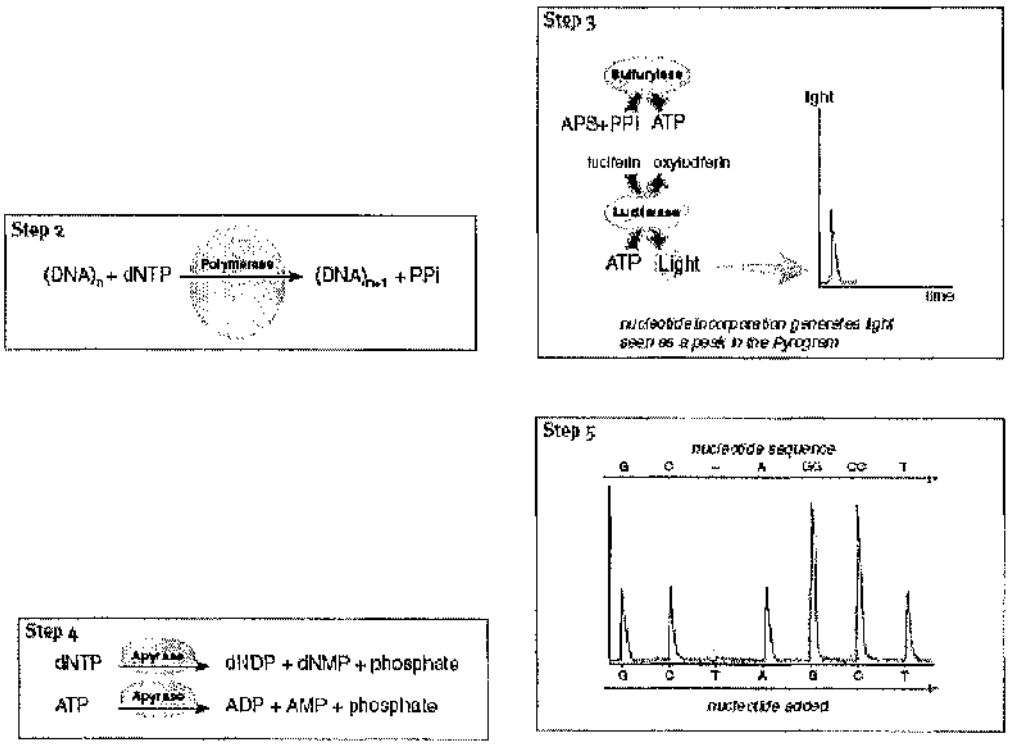


Figure 4.1: The chemistry of Pyrosequencing®, step 2 to step 5. Reproduced with permission of Biotage, Uppsala, Sweden.

4.1.3.3. SNaPshot®

SNaPshot® (Applied Biosystems, Foster City, CA, USA) is a single base extension method of genotyping. It is a consistent and easy to use method with a fairly high throughput of up to 9216 samples in 24 hours using the ABI 3100, 3130 or 3700 PRISM® sequencer (Applied Biosystems). Following amplification of the target DNA sequence, residual primers and unincorporated dNTPs are removed by treatment with shrimp alkaline phosphatase (Exosap). The extension primer then targets the sequence immediately upstream of the SNP site and is extended by a single base in the presence of all four fluorescently labelled dideoxy nucleotides (ddNTPs). Each fluorescent ddNTP emits a different wavelength when excited by the laser and the signal is converted into a specific colour for each base. The size of product is the length of the initial probe plus one fluorescent base. The genotypes are then determined by the colour and location of the peak that is generated by the emitted fluorescence (Pati *et al.*, 2004, Turner *et al.*, 2002).

4.1.4. Preliminary Analysis of Genotyping Data

Before carrying out in-depth analysis or an association analysis, preliminary analyses should be carried out in order to identify areas of concern, for example, missing data or unusual patterns. The 2 most common tests are detailed below.

4.1.4.1. Hardy-Weinberg Equilibrium

In a large, random-mating population where no selection, mutation or migration is occurring, the gene and genotype frequencies are constant from generation to generation and a simple relationship between them can be described. A population in this state is in *Hardy-Weinberg equilibrium* (HWE). The relationship is that if the gene frequencies of 2 alleles of the parents are p and q, then the genotype frequencies of the progeny are p^2, $2pq$ and q^2. In order to test for HWE, a chi-square (X^2) goodness-of-fit test can be used (Balding, 2006, Falconer and Mackay, 1996).

4.1.4.2. Linkage Disequilibrium

Linkage disequilibrium is the non-random association of alleles at 2 or more loci in a population and as stated in Section 5.1 is commonly used in indirect association studies. Linkage disequilibrium provides information on population structure and can also be used to map genes or quantitative trait loci (QTL). Although commonly used in human population studies, it is becoming increasing popular in livestock studies and has been shown to be common and significant in sheep, cattle and pigs (Nsengimana *et al.*, 2004, McRae *et al.*, 2002). Linkage disequilibrium can be measured in a number of ways, the most common being Lewontin's D′ and r^2. Lewontin's D′ has a range of 0 to 1 and was designed for use with loci with 2 or more alleles. It is particularly useful in animal studies due to its ability in handling multi-allelic data. A D′ value of 1 (complete LD) means that at least 1 allele at each locus is completely associated with an allele at the other locus (Devlin and Risch, 1995, Lewontin, 1964). The measurement of r^2 also has a range of 0 to

1 and is the correlation of determination for alleles at 2 loci. An r^2 value of 1 (perfect LD) means that each allele at each locus is completely associated with 1 allele at the other locus and that allele frequency at each loci is identical (Du *et al.*, 2007, Ardlie *et al.*, 2002).

4.1.5. Porcine APP Genes

The four porcine APP genes that this study focused on; *CRP*, *Hp*, *Pig-MAP* and *TTR*, have been previously sequenced and polymorphisms published in some genes, however little further work has been carried out. Each of the genes is briefly discussed below.

4.1.5.1. Haptoglobin

The porcine *Hp* gene (accession no.: AF492467) is located on SSC6 and is 83% homologous to the human haptoglobin α1S. The cDNA is 1182 bp in length with 138 bp of 3' untranslated region (3' UTR). One SNP has been identified in exon 5 of the coding sequence at nt 624 (Ponsuksili *et al.*, 2002). The SNP, a non-synonomous C-T substitution which changes serine to proline, may prove to be of significance in terms of protein function or expression.

4.1.5.2. C-Reactive Protein

The porcine *CRP* gene (accession no.: AB005545) is located on *Sus scrofa* chromosome (SSC) 4 and is 1792 bp (cDNA) long. Two SNP have been reported, the first, a C-T substitution at position nucleotide (nt) 788 rs located within the 3' UTR. The second is a G-A substitution at position nt 1271 also in the 3' UTR (Chomdej *et al.*, 2004).

4.1.5.3. Pig-MAP

The pig-MAP gene or porcine ITIH4 (accession no.: S82800), is located on SSC13 and shows homology (65%) with human ITIH4 (Baskin *et al.*, 1998, Hashimoto *et al.*, 1996). It is 2952 bp (cDNA) in length and contains an *AciI* restriction fragment length polymorphism, however no further details on the precise SNP location are available (Baskin *et al.*, 1998).

4.1.5.4. Transthyretin

The porcine TTR gene (accession no.: X82258), is located on SSC6 and shares 85% homology with human *TTR* gene. The cDNA is 591 bp in length and contains a silent A-T substitution at nt 462 within exon 4 (Archibald *et al.*, 1996, Duan *et al.*, 1995).

The aim of this study was to identify SNP in 4 porcine APP genes; *CRP*, *Hp*, *pig-MAP* and *TTR* and select a manageable number of SNP to be genotyped across the boar population in which APP protein levels had previously been measured. This work was carried out in the Genus, Plc. molecular biology laboratory, Kentucky, USA.

4.2.Materials and Methods

4.2.1. Samples

The tissue samples used in this work were obtained from Genus, Plc. Tail samples were collected from piglets following company protocol and frozen at -20°C. A pool of porcine DNA was also made available for PCR optimisation and SNP discovery. This pool contained DNA from animals of the lines used within this study.

4.2.2. Gene Selection

As previous work in this thesis has centred on the proteins; Hp, CRP, Pig-MAP and TTR, the genes for these four proteins were chosen as candidate genes in order to identify any SNP and possible associations with phenotype, i.e. basal APP concentrations. Each of the four genes has been sequenced in the pig and this data was utilised during primer design. Porcine BAC-end sequences (PigEBAC) with homology to human chromosome 1, (CRP), chromosome 16 (Hp), chromosome 3 (ITIH4 – also referred to as pig-MAP) and chromosome 18 (TTR) were identified via Ensemble (www.ensembl.org/index.html) and this data was also utilized during primer design.

4.2.3. DNA Isolation

DNA was isolated from porcine tail tissue using DNeasy® 96 Tissue kits (Qiagen Inc., Valencia, CA, USA) following the manufacturer protocol. In short, approximately 20 mg

of tissue was cut from each sample and incubated overnight at 55°C in a proteinase K solution to lyse the tissue. Following complete lysis, the DNA was bound to the DNeasy® membrane and washed twice to remove residual contaminants. The purified DNA was eluted twice from the membrane using elution buffer, the first elution yields up to 75% of the DNA from the membrane and the second increases yields by up to 25%. The purified DNA was then frozen at -20°C until use.

4.2.4. SNP Discovery

4.2.4.1. Primer Design

PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and according to the following conditions:

a) the product size was between 450 and 600 bp in length although shorter or longer fragment lengths were used when necessary, for example, spanning an intron
b) the oligonucleotide size was between 20 and 30 nucleotides in length
c) the primers contained approximately equal numbers of each nucleotide and avoided lengths of repeating nucleotides
d) Tm target was 60°C

4.2.4.2. PCR Optimisation

In order to perform SNP discovery, PCR conditions had to be optimised for each set of primers. In order to avoid contamination of samples, stringent conditions were followed during preparation of the PCR. In the Genus laboratory in Franklin, KY, PCR preparation was carried out in a dedicated lab using dedicated instruments and reagents for PCR preparation. Master mixes were used where possible to reduce pipetting steps and potential contamination. Negative controls were included in each reaction using Gibco™ Ultrapure water (QH_2O) (Invitrogen, Carlsbad, CA, USA) instead of DNA. All chemical reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA unless otherwise stated.

4.2.4.3. PCR for Sequencing

Three different optimized PCR conditions were used for preparing the sequencing reaction, they were:

1. A 10 µl reaction containing 1 µl porcine genomic DNA, 0.2 mM each dNTP (Applied Biosystems, Foster City, CA, USA), 1 µl 10x PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 0.5 U Amplitaq Gold® DNA polymerase, and 0.25 mM each forward and reverse primer (Qiagen Operon Inc., Huntsville, AL, USA) was prepared. The mixture was placed in GeneAmp® PCR System 9700 thermal cycler with a heated lid (Applied Biosystems) and underwent initial denaturation at 94°C for 12 min This was followed by 16 cycles of amplification with a denaturation step at 94°C for 30 sec and annealing step for 30

sec. The annealing step used a touchdown program to decrease the temperature from 71°C to 55°C in 1°C steps each cycle. This was followed by an extension step at 72°C for 30 sec. Following the 16 touchdown cycles, 25 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec were performed which were then followed by a final extension step at 72°C for 7 min. The samples were then held at 4°C. Details of primers and expected product sizes for these conditions can be found in Table 4.2.

2. A 10 μl reaction containing 1 μl porcine genomic DNA, 0.2 mM each dNTP, 1 μl 10x PCR buffer, 2.5 mM $MgCl_2$, 0.5 U Amplitaq Gold® DNA polymerase and 0.5 mM each forward and reverse primer was prepared. The mixture was placed in GeneAmp® PCR System 9700 thermal cycler with a heated lid and underwent initial denaturation at 94°C for 12 min. This was followed by 38 cycles of amplification with a denaturation step at 94°C for 30 sec and annealing step at 55°C for 30 sec. This was followed by an extension step at 72°C for 30 sec. Following the 38 cycles, a final extension step at 72°C for 7 min occurred. The samples were then held at 4°C. Details of primers and expected product sizes for these conditions can be found in Table 4.3.

3. As reaction 2 except using an annealing temperature of 65°C for 30 sec. Details of primers and expected product sizes for these conditions can be found in Table 4.4.

Table 4.2: Primers and expected product sizes for optimised PCR conditions 1.

Forward Primer	Sequence (5' to 3')	Reverse Primer	Sequence (5' to 3')	Size
CRP_BES-1F	CTCCTCAGTCCAAGCAAACC	CRP_BES-1R	CCTGTGGCAAGGATACCAGT	523
CRP_BES-2F	CCCTCATTCCCTGATTCTGA	CRP_BES-2R	CCGAATCCTTAACCCACTGA	569
CRP_BES-3F	TTTCCATGGCTCAGGAAACT	CRP_BES-3R	TTGGGGCTTTCACAAATAGG	460
CRP_BES-4F	TTGTAAAGGCGCAGGGTATT	CRP_BES-4R	TGTTGCTGTGGCTATGGTGT	460
CRP_BES-5F	CCATGCAGTACAGTGGGAAA	CRP_BES-5R	CCACAATGGGAACTCCAAAA	532
CRP_BES-6F	GTTCATTTTTGGCCACGTCT	CRP_BES-6R	ATCCCAAAGGCAACATTCTG	488
CRP_BES-7F	GGGGACCCAATAAGCAAAAT	CRP_BES-7R	TCTCTCCATGATGGGTGTCA	283
CRP_BES-8F	TTTTAGGGCCACTCTTGCAG	CRP_BES-8R	CCACAGCAGAACTCCTTCCT	359
CRP_BES-9F	GCTAAGCGTCCTACCTGGAA	CRP_BES-9R	GGGATGATGAGGTCTCCTTG	487
CRP_BES-10F	CTCTGAAGAGGGCTGCTGAC	CRP_BES-10R	TGCAACTAGATCATGCACAAA	494
CRP-1F	AATTCGGCACGAGAGACATC	CRP-1R	ATAGACACGCAGGCACACAG	565
CRP-2F	TTGTCTTCCCCAAAGAGTCG	CRP-2R	CCCACATGTTCACATCTCCA	480
CRP-3F	CTGGGGGCTTTGAGAAGAAC	CRP-3R	TTTAATTGGCCTGGGATGTG	410
CRP-4F	CTGTGCCAAGCAAAGGATCT	CRP-4R	CCTCAGCTGAAGGAATTGGA	298
CRP-5F	ATTCCTTCAGCTGAGGTTGC	CRP-5R	GGGGAGAATGCTGGAAAAAT	454
HP-BES-1F	ACTGGTGGCAAGTCCATTGT	HP-BES-1R	TACACCACAGCCACAGCAAC	214
HP-BES-2F	TGGCTCAGCAGTTGACAAAC	HP-BES-2R	GCAACCTACACCACAGCTCA	464
HP-BES-3F	AACCCTGGCAGCTACACATC	HP-BES-3R	GCATGGTCCTTCCTACCTGA	503
HP-BES-4F	CAGGAGGCTTTTCTCTGCAC	HP-BES-4R	GAGAACCCAGACACCAGAGC	324
HP-BES-5F	TCAGGATTTCTGTGGGAAGG	HP-BES-5R	GGGCTACACCCCAGACTACA	482
HP-BES-6F	AAGTGACCAGAAGCCGAGAA	HP-BES-6R	AACGCCCTATACCTCCCTTG	275
HP-BES-7F	CCGCAGAGTGGGTTAAGGAT	HP-BES-7R	AGGGTCTCTCCTGTGGCATA	460
HP-BES-8F	GTGGGAACTGGGTCCATAAA	HP-BES-8R	CTCCCTTTCCTTCTCCCTTG	512
HP-BES-9F	GCGTGCAAAAGCAAAACATA	HP-BES-9R	CATCATTGGCAGTGCTTTGT	458
HP-BES-10F	TAGAGATCACTGGGCCTGCT	HP-BES-10R	GACCCAGTGGAAGGTTTTGA	522
HP-1F	GGCAGCTTTTTGCAGCAG	HP-1R	CGTGCGCAGTTTGTAGTAGG	426

Forward Primer	Sequence (5' to 3')	Reverse Primer	Sequence (5' to 3')	Size
HP-2F	GCTACGTGGAGCACATGGTT	HP-2R	TGCTTCACATTCAGGCAGTT	900
HP-3F	CTGCCTGAATGTGAAGCAGT	HP-3R	GGTGAGGTTATGGTGGGAG A	153
HP-4F	AGAACCCAGTGGATCAGGTG	HP-4R	ACGGTGCTGCCTTCGTAGTA	496
HP-5F	ACGCCAACCTCAACTTTACG	HP-5R	GGGCTCAAAGGAAGCTCTTT A	492
ITIH4_BES-1F	CCAGGACTTGCACTCCTTTC	ITIH4_BES-1R	TTTTCCCACCAACACTGGTC	447
ITIH4_BES-2F	ATGGGCAGCATCACTTCTCT	ITIH4_BES-2R	ATACCCACTGCCACCTGAAG	479
ITIH4_BES-3F	TTCTTTTTATGGTCGCACCTG	ITIH4_BES-3R	TTTTTGCTTCATCCCAGAGA	481
ITIH4_BES-4F	TGCAAAGAAGTTCATTCTGTC CT	ITIH4_BES-4R	TTTATCCCTTCAAAGGTTCA AAT	578
ITIH4_BES-5F	CACACCTGTGGCCCTAAAAA	ITIH4_BES-5R	TTGATTTTTCCTTGCTGGGT A	467
ITIH4_BES-6F	TCTCCCTGCATCCTGTTTTC	ITIH4_BES-6R	TTGGCTGAAGTCAAGACTCG	464
ITIH4_BES-7F	AGGGAGTGCTGTCCAGAGAA	ITIH4_BES-7R	CATTACGGAGCAGTGAAGC A	586
ITIH4_BES-8F	CAGAATTCCCAACCACGTCT	ITIH4_BES-8R	ACCAAGCGGCTCAGATAAA A	478
ITIH4_BES-9F	CTTGGATCCCAAATTGCTGT	ITIH4_BES-9R	CTGGCTCTGTGGGTTAAGGA	539
ITIH4_BES-10F	GGCAGAGAAGAGGGAGACCT	ITIH4_BES-10R	CAGGGTGGAAAGGTTAGCA G	493
ITIH4-1F	GTGGACTCCAAGGTCTCGTC	ITIH4-1R	GGTGATGAAAGCCTTCTTGG	123
ITIH4-2F	ATCGATGGTGTGACCTACCC	ITIH4-2R	CCTCTGCCAGTTCATTGGTC	622
ITIH4-3F	GGCATCAGCTTTCTGGAGAC	ITIH4-3R	TGTCCGGTTCACATCATAGC	718
ITIH4-4F	GATTCAAGCCGACACTCTCC	ITIH4-4R	GGATTTTCCTGCCCCTCAT	1158
ITIH4-5F	CGGAGGTCTGGTCTGCAATA	ITIH4-5R	ATCGGTGAGGAGGATGATG A	717
ITIH4-7F	TCCTGAGCCCCAAGTACATC	ITIH4-7R	GTTCTTGGCCTTCAGGTTTG	409
ITIH4-8F	GAGCCCTGAGCTTGTCACTC	ITIH4-8R	GAGTGGGTGTTCCCCTGTC	452
ITIH4-9F	GACAGGGGAACACCCACTC	ITIH4-9R	TGGATGTTCTGTCTCCCACA	366
ITIH4-10F	TGTGGGAGACAGAACATCCA	ITIH4-10R	TACGGGAAAACGGATGAGA A	555
ITIH4-11F	AAGGACAAGCCCAAGGTTTT	ITIH4-11R	TCATGTGACGTTTCATCAGG	1016
ITIH4-12F	GAGGTGCTATCCGTTCCTGA	ITIH4-12R	TGCTTGAGGTCCACACAGAG	421
ITIH4-13F	CAGCTCTGTGTGGACCTCAA	ITIH4-13R	TCCTTCCACTTGTACGCAGA	1208
ITIH4-14F	TGGCCACTATGAGAGGGAGA	ITIH4-14R	CCGGGCATCACTGAGTAGA G	162
ITIH4-15F	GACAAGGCGGGACTTCTTCT	ITIH4-15R	CACTGTTCGCTTGCTGTCAT	341
ITIH4-16F	ATGACAGCAAGCGAACAGTG	ITIH4-16R	ATCTCCCCATGATGTTCCAG	714
TTR_BES-1F	ACTAGCCTGGGAACCTCCAT	TTR_BES-1R	AAGCCCTGGAAGTTGAGAC A	483
TTR_BES-2F	TGCATCTTTCTGCTGGACTG	TTR_BES-2R	GTGTGCCTCAGCTTTCTTCC	476
TTR_BES-3F	CCAGTTGTGGCTCAGCAGTA	TTR_BES-3R	AAGCAGCCTTCACAGAGAC C	458
TTR_BES-4F	ATGCAGAAAAAGCATTTGAC A	TTR_BES-4R	GTCTCTTCAGCAAGTGGTGC T	450

Forward Primer	Sequence (5' to 3')	Reverse Primer	Sequence (5' to 3')	Size
TTR_BES-5F	ATGGCTGGGTCACTTTTCTG	TTR_BES-5R	TCTGAGCTCCTCCCACAACT	355
TTR_BES-6F	TGGCCCATAGACACGTTACA	TTR_BES-6R	ACGACGTGCGTAAGGAGAG A	505
TTR_BES-7F	TTCTCCTGCCTTCTCCTTTG	TTR_BES-7R	CTCCCATAGGCTGGATGAAA	482
TTR_BES-8F	TCTCCCTACTCCCGATGGAT	TTR_BES-8R	ATGCAGTCCCTTTGAGGAGA	588
TTR_BES-9F	AATTTTGCACGTCTAGCCTCT	TTR_BES-9R	TCTTACCATGGTTTGCCACT T	294
TTR_BES-10F	GTGAGGACCACGAGTCAGGT	TTR_BES-10R	TGCCCTTCATTTCTGTTTCC	503
TTR-1F	GATGGCTTCTTACCGTCTGC	TTR-1R	GCATCCAGGACTTTGACCAT	1041

Table 4.3: Primers and expected product sizes for optimized PCR conditions 2

Forward Primer	Sequence (5' to 3')	Reverse Primer	Sequence (5' to 3')	Size
TTR-2F	TGCTGGTGAATCCAAGTGTC	TTR-2R	CAAAGGGCTCCCAAGTCC	122
TTR-3F	AAAACCAGTGAATTTGGAGAGC	TTR-3R	ATTCATGGAATGGGGAAATG	127
TTR-4F	TGTGTTCACAGCCAACGACT	TTR-4R	TTGTCTCTGCCCGAGTTTCT	212

Table 4.4: Primers and expected product sizes for optimized PCR conditions 3

Forward Primer	Sequence (5' to 3')	Reverse Primer	Sequence (5' to 3')	Size
ITIH4-6F	GTGCAGCTGCTGGAAAGAG	ITIH4-6R	CTTTGAAGAACAGCCGGAAG	653

4.2.4.4. PCR Product Assessment

PCR products were checked on a 3% super fine resolution (SFR) agarose electrophoresis gel stained with ethidium bromide (Amresco, Solon, OH, USA) and UV light was used for product visualisation using an AlphaImager™ (Alpha Innotech Corporation, San Leandro, CA, USA). An aliquot of the PCR product was mixed with 3 µl loading dye and applied to the gel which was electrophoresed at 150V for 30 min. The molecular weights of the fragments were estimated by running a 100 bp DNA molecular weight ladder (New England BioLabs® Inc., Ipswich, MA, USA) alongside the PCR products.

4.2.4.5. Sequencing Reaction

Following DNA amplification, excess PCR primers and dNTPs were degraded by adding 2 µl Exosap-IT® (USB® Corporation, Cleveland, OH, USA) and incubating for 1 hr at 37°C and 15 min at 72°C. Three microlitres of the cleaned PCR product was then mixed with 4 µl Big Dye® V3.1 mix (Applied Biosystems), 2.36 µl QH_2O and 3.2 mM of the appropriate primer. This was placed in a thermal cycler and underwent 30 cycles of 96°C for 10 sec, 55°C for 5 sec and 60°C for 4 min. The samples were then held at 4°C. Sequencing reactions were set up from both the forward and reverse PCR primers.

The products were purified using 10 µl Cleanseq® magnetic beads (Agencourt Bioscience Corporation, Beverly, MA, USA). The products bound to the beads and they were washed twice with 73% ethanol (73 µl for 3 min and 100 µl for 30 sec). The ethanol was aspirated

off and excess ethanol allowed to evaporate. A 40 µl aliquot of 0.1 mM EDTA was added to elute the products from the beads. Twenty microlitres of each sample was then loaded onto an ABI 3100 automated sequencer (Applied Biosystems) and analysed using Sequencher™ software (Gene Codes Corporation, Ann Arbor, MI, USA).

4.2.5. Genotyping

Single nucleotide polymorphisms were identified by the Sequencher™ software and manually checked. The choice of SNP to be genotyped was based upon the degree of heterozygosity shown by the multiple peaks on the chromatogram and the position of the SNP i.e. exonic versus intronic. Three methods of genotyping were then used. The choices of method were based on the suitability and ease of genotyping the SNP in question with that technique.

4.2.5.1. PCR-Restriction Fragment Length Polymorphism

Restriction enzymes were chosen using NEBcutter V2.0 software (http://tools.neb.com/NEBcutter2/index.php) and obtained from New England BioLabs® with the associated buffers. Where possible, enzymes were chosen that cut at only at the polymorphic site.

PCR was carried out (using porcine genomic DNA samples) and the PCR products checked by visualisation on 3% SFR agarose electrophoresis gel stained with ethidium bromide as

described previously. The products were then digested using the appropriate restriction enzyme (see Table 4.5) and with the recommended buffer and incubation times. Following incubation, 3 µl of loading dye were added to the digested products and 6 µl of each sample ran on a 3% SFR agarose electrophoresis gel stained with ethidium bromide for approximately 30 min at 150V and UV light used for product visualisation. The molecular weights of the fragments were estimated by running a 100 bp DNA molecular weight ladder alongside. Genotype scoring was then performed manually.

Table 4.5: SNP and PCR-RFLP assay details

Gene	SNP	SNP Type	Enzyme	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Allele 1 (bp)	Allele 2 (bp)
CRP	FSAD0003	Y	BsaJI	CTGGGGGCTTTGAGAAGAAC	TTTAATTGGCCTGGGATGTG	396, 14	223, 173, 14
CRP	FSAD0013	Y	ScrFI	GTTCATTTTTGGCCACGTCT	ATCCCAAAGGCAACATTCTG	226, 191, 71	226, 112, 79, 71
Hp	FSAD0014	M	BbsI	TAGAGATCACTGGGCCTGCT	GACCCAGTGGAAGGTTTTGA	522	393, 129
Hp	FSAD0016	Y	AluI	GTGGGAACTGGGTCCATAAA	CTCCCTTTCCTTCTCCCTTG	268, 159, 84	159, 135, 133, 84
Hp	FSAD0024	R	PflfI	GGCAGCTTTTTGCAGCAG	CGTGCGCAGTTTGTAGTAGG	435	327, 108
ITIH4	FSAD0027	R	NlaIII	GGCATCAGCTTTCTGGAGAC	TGTCCGGTTCACATCATAGC	314, 223, 150	314, 167, 150, 56
ITIH4	FSAD0031	Y	BseRI	GGCATCAGCTTTCTGGAGAC	TGTCCGGTTCACATCATAGC	352, 179, 156	352, 156, 132, 47
ITIH4	FSAD0035	Y	HinfI	ATGACAGCAAGCGAACAGTG	ATCTCCCCATGATGTTCCAG	669	486, 183
TTR	FSAD0038	W	AluI	TGTGTTCACAGCCAACGACT	TTGTCTCTGCCCGAGTTTCT	212	107, 105

4.2.5.2. Pyrosequencing®

PCR and sequencing primers were designed using PSQ Assay Design Software V1.0 according to the manufacturer's guidelines and primers were supplied by Integrated DNA Technologies Inc. (IA, USA). One of each set of PCR primers must be HPLC purified and biotinylated (see Table 4.6 for primers).

A 25 µl reaction containing 2 µl porcine genomic DNA, 0.5 mM each dNTP, 2.5 µl 10x PCR buffer, 3.75 mM $MgCl_2$, 1.25 U Amplitaq Gold® DNA polymerase and 0.5 mM each forward and reverse primer was set up. The mixture was placed in GeneAmp® PCR System 9700 thermal cycler with a heated lid and underwent initial denaturation at 94°C for 10 min. This was followed by 20 cycles of amplification with a denaturation step at 94°C for 30 sec and annealing step for 30 sec. The annealing step used a touchdown program to decrease the temperature from 62°C to 52°C in 0.5°C steps each cycle. This was followed by an extension step at 72°C for 30 sec. Following the 20 touchdown cycles, 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 77°C for 30 sec were performed which were then followed by a final extension step at 72°C for 7 min. The samples were then held at 4°C before being checked as previously.

The resulting PCR product, with one biotinylated strand, was mixed with 45 µl binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% w/v Tween 20, pH 7.6) and 3 µl of Streptavidin Sepharose™ beads (Amersham Biosciences, Piscataway, NJ, USA) and agitated for 15 min at room temperature to allow immobilization of a single stranded

template to the beads. In the meantime, 0.16 µl of sequencing primer (100 mM stock solution) was added to 39.84 µl annealing buffer (20 mM Tris-Acetate, 2 mM $MgAc_2$, pH 7.6) and a PSQ 96 well plate prepared with the solution. Following agitation, a Vacuum Prep Tool™ (Biotage Inc., Foxboro, MA, USA) was used to capture the beads and wash with 70% ethanol for 5 seconds, denaturation solution (0.2 M NaOH) for 5 seconds and wash buffer (10 mM Tris-Acetate, pH 7.6) for 5 sec. The vacuum was removed and the beads released into the sequencing primer/annealing buffer solution before being heated to 80°C for 2 min and cooled to room temperature.

Pyrosequencing® was performed at room temperature using an automated PSQ 96 MA and the associated software (Biotage Inc.). A preprogrammed sequence of the nucleotides dATP, dGTP, dCTP and dTTP was added in the presence of polymerase, sulfurylase, apyrase and luciferase (PyroGold Reagents, Biotage Inc.). The genotype scoring was done using PSQ 96 SNP software with default parameters set.

4.2.5.3. SNaPshot®

PCR and internal primers as described in Table 4.7 were designed using Primer3 software and primers were supplied by Sigma-Proligo (TX, USA).

PCR was carried out with the optimised conditions. Following DNA amplification, excess PCR primers and dNTPs were degraded by adding 2 µl Exosap-IT® and incubating for 45 min at 37°C and 15 min at 80°C. The SNaPshot® reaction was then set up on ice as

follows; 1 μl SNaPshot® multiplex ready reaction mix (Applied Biosystems), 0.5 μl 20X SNaPshot® buffer (400 mM Tris-Base, 10 mM $MgCl_2$, pH 9), 3 μl 0.2 μM extension primer, 1.5 μl PCR product, 4 μl QH_2O. The mixture was placed in GeneAmp® PCR System 9700 thermal cycler with a heated lid and underwent 20 cycles of amplification with a denaturation step at 96°C for 10 scc and annealing step of 50°C for 30 sec. This was followed by an extension step at 60°C for 30 sec. Post-extension products were then purified using 0.4 μl CIP (New England BioLabs® Inc.), 0.2 μl Buffer 3 (New England BioLabs® Inc.) and 0.9 μl QH_2O with a 1 hr incubation of 37°C followed by 75°C for 15 min.

The final product was diluted 1:2 with QH_2O and 1 μl of the diluted final product was denatured with 9.4μl Hi-Di™ Formamide (Applied Biosystems) and 0.15μl 120LIZ® size standard (Applied Biosystems) at 95°C for 5 min before being placed on ice for at least 2 min. The fragments were then run on an ABI PRISM 3100 DNA automated sequencer and analysed using GeneMapper® V3.5 software (Applied Biosystems).

Table 4.6: Pyrosequencing® Primers (Biotinylated primer is indicated in bold)

Gene	SNP	SNP Type	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Internal primer (5' to 3')
CRP	FSAD0006	Y	GCCAAGCAAAGGATCTTATACAGG	***CCACACCCCCATCAGATTTA***	GATCTTATACAGGGCTGC
Hp	FSAD0025	S	***ACCCCAGCACTGCCAGAT***	GGCTGTGTCCCCAGTATTCCT	CAGAGCTTAGGAGGGAA
Hp	FSAD0026	R	***CCAAGGTGTACACTCCTGAAACAA***	CGCACCAGGTCCAGATACAA	TAGGCTCATTTCTTTCC
ITIH4	FSAD0034	R	***GCGTGCTAGAAAGGGAAACCT***	GGCTACGTCCGAGTCCAGATT	CTATCTTGGCTTTGGG

Table 4.7: SNaPShot® Primers

Gene	SNP	SNP Type	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Internal primer (5' to 3')
TTR	FSAD0037	K	TCTGAAGGTGCATGTCAACAG	TCTGCCATGAGATCCAAACA	GTGATCCCTTGGCTCTGCT AATAATAAT
TTR	FSAD0039	K	ACCAGCGATAATGGGAAAAA	CAGCTCTGGAACCTGGTCTT	CCTCTCCTGCTTTATGCAA CTTCTAG

4.2.6. Genotyping Assessment

In compliance with Genus, Plc. protocol, the genotyping scores for each SNP were validated by two people.

4.2.7. Statistical Analysis

Genotype frequency was analysed using the PROC FREQ program and linkage disequilibrium and associated measurements were analysed using the PROC ALLELE program of SAS 9.1 (The SAS Institute, Cary, NC, USA).

4.3.Results

4.3.1. SNP Discovery

A total of 71 SNP were discovered from the pool of genomic DNA as shown in Table 4.8. Twenty-nine were found in the CRP gene, 13 in the Hp gene, 26 in the Pig-MAP (ITIH4) gene and 3 in the TTR gene.

Table 4.8: SNP discovery (SNP is indicated in bold)

Gene	SNP	Forward Primer	SNP Sequence
CRP	FSAD0001	CRP-2F	GTGGTGCCGATGTC**R**TTTTCAAGCCTCATCAGAGT
CRP	FSAD0002	CRP-2F	CCTCATCAGAGTTC**Y**GAACCCATGCACTTCTGTAT
CRP	FSAD0003	CRP-3F	TCCCAAAGGTGCCT**Y**CTGGGGTTACACC
CRP (BAC END)	FSAD0004	CRP_BES-1F	TTACTTGGTTTTAT**R**ACACTTACATTTGCATGACT
CRP (BAC END)	FSAD0005	CRP_BES-3F	TGACAGGGCCATAT**W**AATGACCTATTTGTGAAAGC
CRP	FSAD0006	CRP-4F	TATACAGGGCTGCA**Y**ATTAGAGCTAGCAGGCCACA
CRP	FSAD0007	CRP-4F	GACACTCAAGCATC**K**ATGGGTTTTTG
CRP	FSAD0008	CRP-4F	TGTTTTGGCCGCAC**Y**TGCAGCATGTGGAAGTTC
CRP	FSAD0009	CRP-4F	GCAGTGACAAGGCC**R**GATCCCCCGCCTGCTGCAAC
CRP (BAC END)	FSAD0010	CRP_BES-6F	TTGGCCACGTCTGA**K**GCATGTGGAAGCTCCTAGG
CRP (BAC END)	FSAD0011	CRP_BES-6F	TGGAAGCTCCTAGG**Y**CGGAGAGTGCACACAC
CRP (BAC END)	FSAD0012	CRP_BES-6F	AGTCCCCACTGTGG**Y**TCAGTGGATTAAGAATTCGA
CRP (BAC END)	FSAD0013	CRP_BES-6F	TCGACTGCACCAGC**Y**CGGCTTGCTGTGAAGGCACA
Hp (BAC END)	FSAD0014	HP_BES-10F	TCTTGGGCCACCGT**M**TTCTCTYGCCTGGACTCCCT
Hp (BAC END)	FSAD0015	HP_BES-10F	CCACCGT**M**TTCTCTYGCCTGGACTCCCTG
Hp (BAC END)	FSAD0016	HP_BES-8F	ATATTTATTTCAGC**Y**CTCCTTGTGCATAATTGTCTG
Hp (BAC END)	FSAD0017	HP_BES-6F	AAAGGCATGCACCT**R**GTCAGGAACGCTCACTGAGGA
CRP	FSAD0018	CRP-5F	CATCGTGGCTCAGC**S**GAAACAAATCTGTCTAGGAAC
CRP	FSAD0019	CRP-5F	AACATTTTTATTTC**Y**TTTTATAGGCACATCCACCCC
CRP (BAC END)	FSAD0020	CRP_BES-10F	GGGAACAGATAGCC**Y**WACCTTTCTATCTTCTCATC
CRP (BAC END)	FSAD0021	CRP_BES-10F	GGAACAGATAGCC**Y**WACCTTTCTATCTTCTCATCC
CRP (BAC END)	FSAD0022	CRP_BES-9F	TGCATAGCCCCAGT**K**TSCAAACAGTACACCTTGTTT
CRP (BAC END)	FSAD0023	CRP_BES-9F	CATAGCCCCAGTK**T**SCAAACAGTACACCTTGTTTTT
Hp	FSAD0024	HP-1F	CCCACCCGGACCCT**R**TCGGATCTGTTACCCCTAT
Hp	FSAD0025	HP-1F	AAGAGCCAACTGCT**S**CTTCCCTCCTAAGCTCT
Hp	FSAD0026	HP-2F	GCTCATTTCTTTCC**R**TTTGTTTCAGGAGTGTACACC
ITIH4	FSAD0027	ITIH4-3F	TGGAATTCCCTAGC**R**TGGGACCCTTGGK**C**AGAATC
ITIH4	FSAD0028	ITIH4-3F	CRTGGGACCCTTGG**K**CAGAATCTCTGAGCTGGAGGS
ITIH4	FSAD0029	ITIH4-3F	CTCTGAGCTGGAGG**S**ACCTGGGAGGTTCCTGTG
ITIH4	FSAD0030	ITIH4-3F	GACTATTATGAAAG**Y**CCCAGAACCAGTTAGGAAGG
ITIH4	FSAD0031	ITIH4-3F	GGATTCACTCC**Y**CTACTACCCCGAAATTGAGGCAGT
ITIH4	FSAD0032	ITIH4-3F	GGAATTCTCTCTTC**R**GATGGGGAGGAGTGGTGGCAT

Gene	SNP	Forward Primer	SNP Sequence
ITIH4	FSAD0033	ITIH4-11R	ATTGGATTTGTTACWCTCTGGAGGCTGGCTCAGGATG
ITIH4	FSAD0034	ITIH4-11R	GAAACCTTCCTCCRTTCCCAAAGCCAAGATAGT
ITIH4	FSAD0035	ITIH4-16F	TAGACAGTTCTGACYCAGCTCTCAGAGAC
ITIH4 (BAC END)	FSAD0036	ITIH4_BES-2F	CACAGAGGCGCCTAYGGACGCCCTCTACCTTGT
TTR	FSAD0037	TTR_BES-7F	CTGCTAATAATAATKTTGGCCCCATAATATTCGTG
TTR	FSAD0038	TTR-4F	CCCAAGGAGGGAGCWCTTTGAAGGACAAGGGACAG
TTR (BAC END)	FSAD0039	TTR_BES-10F	TATCAGAGTGAGAGKCTAGAAGTTGCATAAAG
ITIH4 (BAC END)	FSAD0040	ITIH4_BES-5F	CACTCACTCAAATCYAGGAAGCACAACGAGTACCAT
ITIH4 (BAC END)	FSAD0041	ITIH4_BES_7F	CTATTGCCTACTCTRGGGGGCTCTAGGCCT
ITIH4 (BAC END)	FSAD0042	ITIH4_BES_7F	GAGAGGCACCAGGCYGCCACCCAGAATTTGCTT
ITIH4 (BAC END)	FSAD0043	ITIH4_BES-4F	ATTCTGGAATTTACRGTGCTTAGGACAGACATCCATTT
HP	FSAD0044	HP-4F	CATAACCTCACCTCRGGGGCCACGCTCATCAATGAACA
HP	FSAD0045	HP-4F	ACAAAAGCAAAAGAYATTGCTCCTACTTTAAGAC
HP	FSAD0046	HP-5F	GAGCACACCTTCTGYGCCGGCTTGTCCAAGTACCAGGA
ITIH4	FSAD0047	ITIH4-5F	AGAGTATGGGGCAGSTCCTGCCTCTAGAGCATTC
CRP (BAC END)	FSAD0048	HP_BES-5F	AGCTCCCTGGTATCYAAAAGAAAAGGCCCACCCCTCA
CRP (BAC END)	FSAD0049	HP_BES-5F	GCTTGTCCCAGGCARCAAGCAGGAAACAATC
CRP (BAC END)	FSAD0050	HP_BES-5F	GGTCATCACAGGAGKCAAGGGTTACAAAGGCCTGGCCC
CRP (BAC END)	FSAD0051	CRP_BES-2F	GTGGAAGTTCCAATMTGGGACAATAGATAGAGA
CRP (BAC END)	FSAD0052	CRP_BES-2F	ACTTTGGGAGCTCAYTGTAAGCCAATAATTGC
CRP (BAC END)	FSAD0053	CRP_BES-2F	ATATGCCTCAGGTCMTTGGGTGGAGGATGGGGTGAGGC
CRP (BAC END)	FSAD0054	CRP_BES-2F	TCAGAGGCACAAAGMAYCTGAACCTCGGTCAGTC
CRP (BAC END)	FSAD0055	CRP_BES-2F	AGAGGCACAAAGMAYCTGAACCTCGGTCAGTC
CRP (BAC END)	FSAD0056	CRP_BES-2F	CTCGGTCAGTCCAGRCACTGGGTGAGTGATTCTAAT
CRP (BAC END)	FSAD0057	CRP_BES-2F	GAACCATGAGGTTGMGGGTTTGATCCCTGGCCTTG
ITIH4	FSAD0058	ITIH4 11F	CTCCRTTCCCAAAGMCAAGATAGTCCCTCCAGCTGTTTCT
Hp (BAC END)	FSAD0059	HP_BES-6F	GCGCCCACCTCCTTYTCGTCCGCAAGGAGGTTCT
ITIH4	FSAD0060	ITIH4-3F	AGTGGTGGCATTGTYATGGCCTCGCTGCGTCTCCTAA
ITIH4	FSAD0061	ITIH4 7F	CTGCAAGGGAGGGARACTCTGCCTGGCCACCCAGGAACC
ITIH4	FSAD0062	ITIH4 7F	GTCACCCCTCTCACRTCTATGGTGATCACCAAACC
ITIH4	FSAD0063	ITIH4-10F	GCACTAGAACTGGGAGTGGGGTRGGGAG
ITIH4	FSAD0064	ITIH4-10F	GGGAGRAAAGCCACT
ITIH4	FSAD0065	ITIH4-10F	AAAGCCACTRCTCCAGCAGGGAGGAG
ITIH4	FSAD0066	ITIH4-10F	AGCAGGGAGGAGTTGAAGARGGGTCTGTC
ITIH4	FSAD0067	ITIH4-10F	GGGTCTGTCRCTCCAGGCTTGAATTTCTCCCAGAGTAAT

Gene	SNP	Forward Primer	SNP Sequence
ITIH4	FSAD0068	ITIH4-10F	GGCTTTGGGGACCCWTGACACTGTTTGAGGGAAGACTG
ITIH4	FSAD0069	ITIH4 9F	TCCAGAAACGTGCCRCCAAGAAGCAAACTCAGAT
Hp (BAC END)	FSAD0070	HP_BES-5F	GGCATTGTAATCTASAGGACAACTCTGTATACATTTCCCC
Hp (BAC END)	FSAD0071	HP_BES-10F	AGGTGGGCCTTTAARAGTGCAAGTCAGATCTTGTTA

4.3.2. Genotyping

Seventeen SNP were chosen as the best candidates for genotyping across the boar samples; of these, 5 were found in the Hp gene, 5 in the Pig-MAP gene, 4 in the CRP gene and 3 in the TTR gene.

4.3.2.1. Genotyping of Hp SNP

Of the 5 SNP genotyped in the Hp gene, 3 were intronic polymorphisms. These were: FSAD0024 (A-G substitution) genotyped by PCR-RFLP, FSAD0025, (G-C substitution) and FSAD0026, (A-G substitution), both genotyped by Pyrosequencing®. Two SNP were identified using BES data; FSAD0014, (A-C substitution) and FSAD0016 (C-T substitution), and were genotyped using PCR-RFLP. Examples of genotype scoring are shown in Figure 4.2.

4.3.2.2. Genotyping of CRP SNP

Four SNP were genotyped in the CRP gene with 2 located in the 3' untranslated region. FSAD0006 (C-T substitution) was located at nt 960 of the gene sequence and FSAD0003 (C-T substitution) at nt 808, genotyped using Pyrosequencing® and PCR-RFLP, respectively. Two SNP were identified using BES data; FSAD0013 (C-T substitution) and FSAD0020 (C-T substitution), and were genotyped using PCR-RFLP and SNaPshot®, respectively. Examples of genotype scoring are shown in Figure 4.3.

4.3.2.3. Genotyping of Pig-MAP SNP

Five SNP were genotyped in the Pig-MAP gene; 4 were intronic polymorphisms. These were as follows; FSAD0027 (A-G substitution), FSAD0031 (C-T substitution) and FSAD0035 (C-T substitution), all genotyped using PCR-RFLP and FSAD0034 (C-T substitution) genotyped using Pyrosequencing®. One SNP was identified using BES data, FSAD0036 (C-T substitution) and it was genotyped by Pyrosequencing®. Examples of genotype scoring are shown in Figure 4.4.

4.3.2.4. Genotyping of TTR SNP

Three SNP were genotyped in the TTR gene. FSAD0038 (a silent A-T substitution) was located at nt 462 (in exon 4 of TTR) and genotyped using PCR-RFLP. The remaining 2 SNP were identified using BES data; FSAD0037 (G-T substitution) and FSAD0039 (G-T substitution) which were genotyped by SNaPshot®. Examples of genotype scoring are shown in Figure 4.5.

Figure 4.2: Genotype scoring of SNP found in the Hp gene

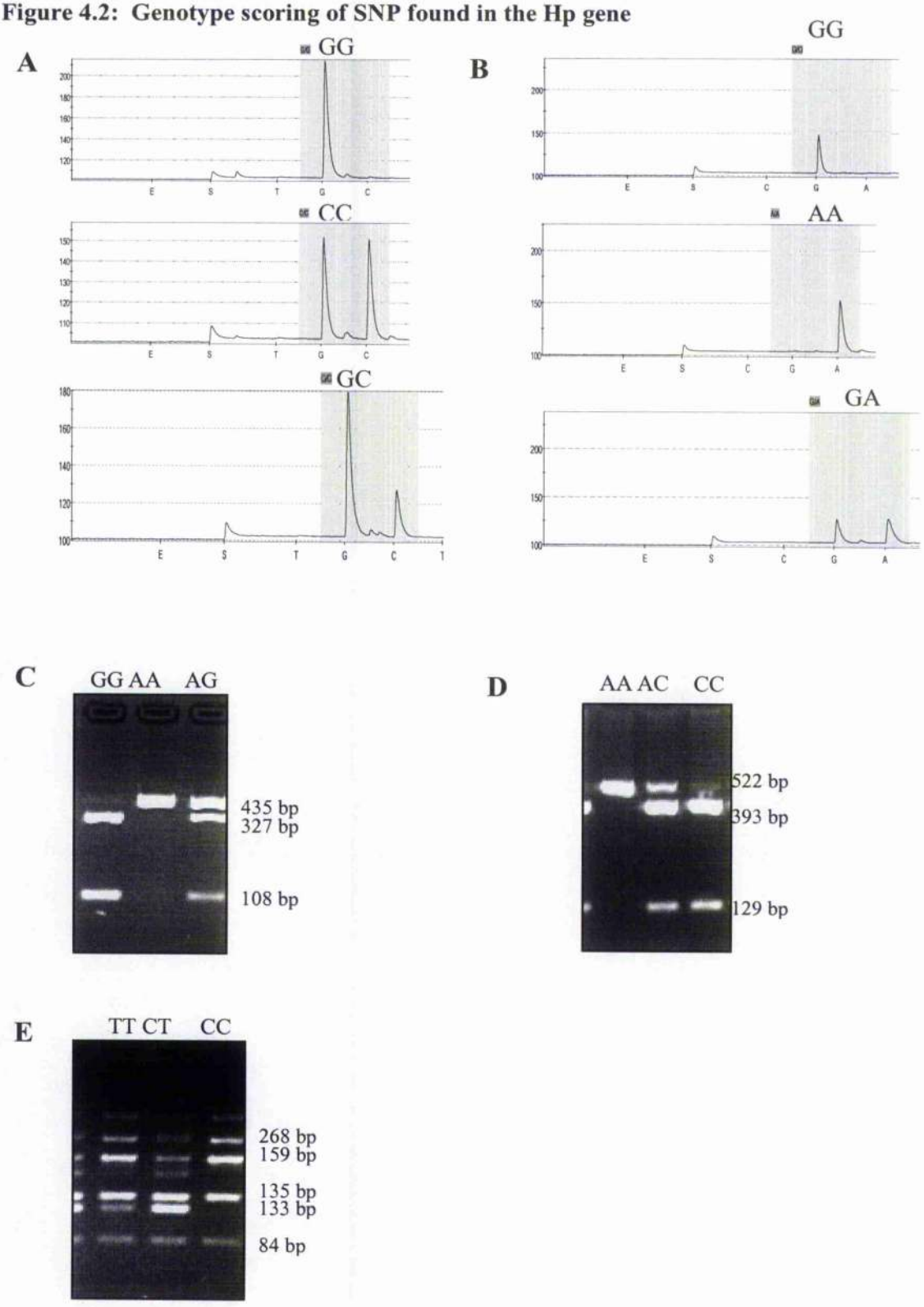


Figure 4.2: Genotype scoring of SNP found in the Hp gene. **A**: FSAD0025; pyrogram™ showing GC polymorphism (indicated above trace) In this instance, the SNP is preceded by two G nucleotides, this gives rise to a double peak for G in the CC genotype and a triple peak for G in the GC genotype. **B**: FSAD0026; pyrogram™ showing AG polymorphism (indicated above trace). **C**: FSAD0024; PCR-RFLP showing AG polymorphism (digestion by *PflFI*, position of relevant bands indicated). **D**: FSAD0014 showing AC polymorphism (digestion by *BbsI*, position of relevant bands indicated). **E**: FSAD0016 showing CT polymorphism (digestion by *AluI*, position of relevant bands indicated).

Figure 4.3: Genotype scoring of SNP found in the CRP gene.

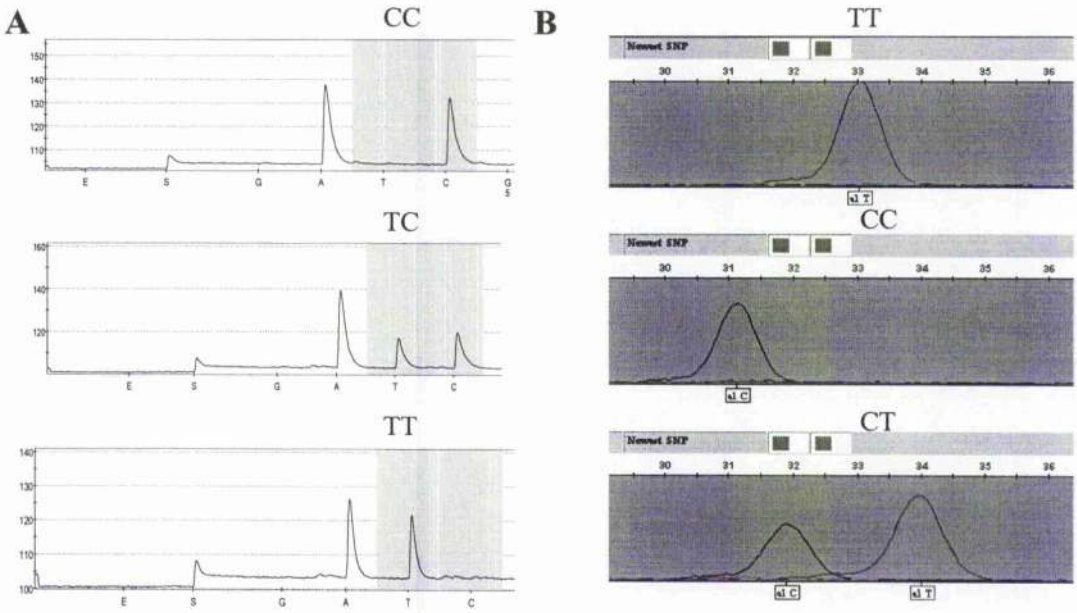


Figure 4.3: Genotype scoring of SNP found in the CRP gene. **A**: FSAD0006; pyrogram™ showing CT polymorphism (indicated above trace). **B**: FSAD0020; SNaPshot® output showing CT polymorphism (indicated above trace). **C**: FSAD0003; PCR-RFLP showing CT polymorphism (digestion by *BsaJI*, position of relevant bands indicated). **D**: FSAD0013 showing CT polymorphism (digestion by *ScrFI*, position of relevant bands indicated).

Figure 4.4: Genotype scoring of SNP found in the Pig-MAP gene.

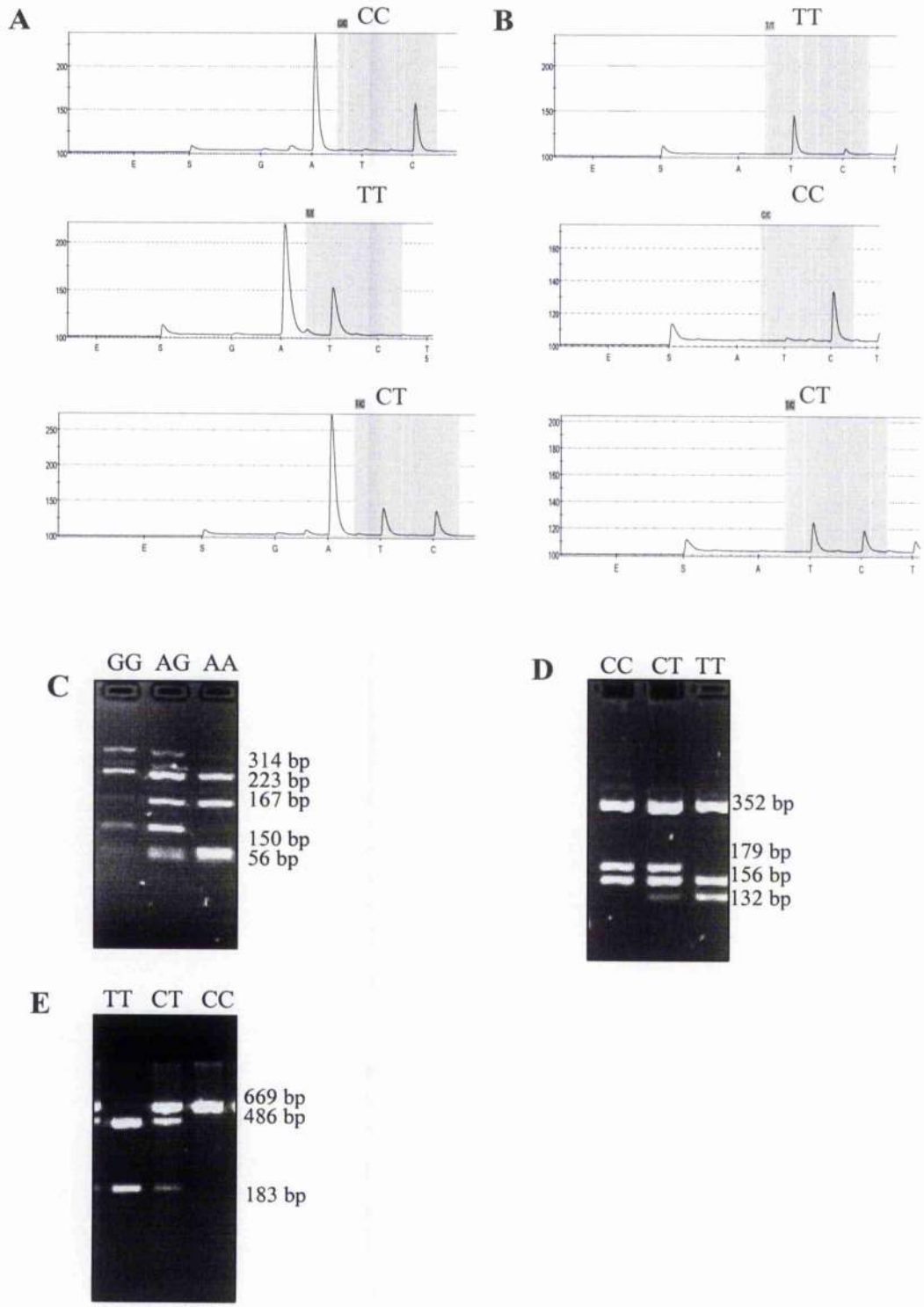


Figure 4.4: Genotype scoring of SNP found in the Pig-MAP gene. **A**: FSAD0034; pyrogram™ showing CT polymorphism (indicated above trace). **B**: FSAD0036; pyrogram™ showing CT polymorphism (indicated above trace). **C**: FSAD0027; PCR-RFLP showing AG polymorphism (digestion by *NlaIII,* position of relevant bands indicated). **D**: FSAD0031; PCR-RFLP showing CT polymorphism (digestion by *BseRI*, position of relevant bands indicated). **E**: FSAD0035; PCR-RFLP showing CT polymorphism (digestion by *HinfI*, position of relevant bands indicated).

Figure 4.5: Genotype scoring of SNP found in the TTR gene

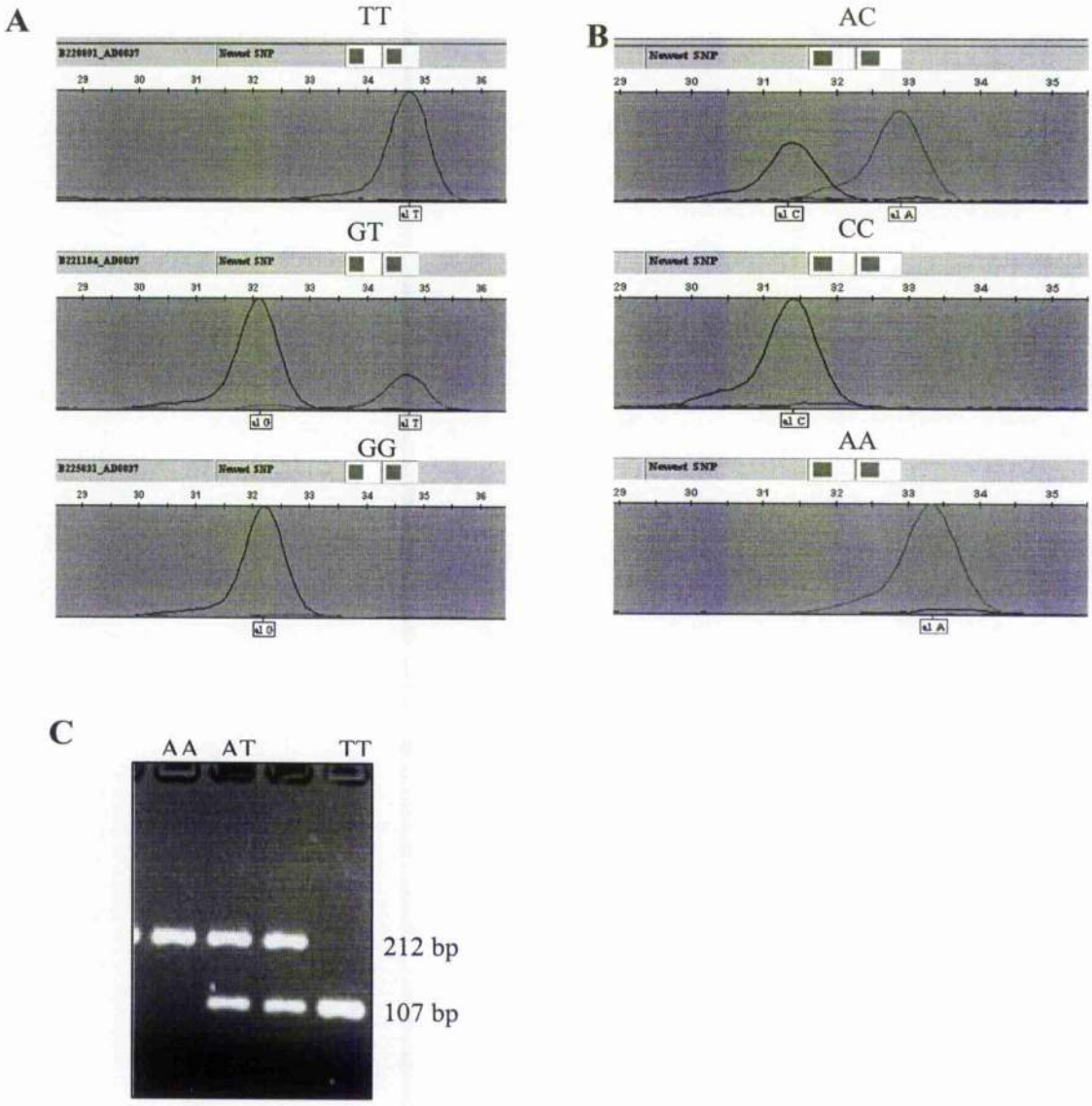


Figure 4.5: Genotype scoring of SNP found in the TTR gene. **A**: FSAD0037; SNaPShot® output trace showing GT polymorphism (indicated above trace). **B**: FSAD0039; SNaPShot® output trace showing CA polymorphism (indicated above trace). **C**: FSAD0038; PCR-RFLP showing AT polymorphism (digestion by *AluI*, position of relevant bands indicated).

4.3.3. Allele Frequency

Allele frequency was calculated for each SNP in each of the lines used in this study (Tables 4.9 – 4.12). Allele frequencies showed normal variability in all genes, however in all 4 genes, mono-allelic SNP were found within individual lines. In the Hp gene, mono-allelic SNP were found in Line C for FSAD0024, FSAD0025 and FSAD0026 and in Line E for FSAD0024 and FSAD0025. In the CRP gene, mono-allelic SNP were found in Line C and Line E for FSAD0006. In the Pig-MAP gene, FSAD0034 was mono-allelic in Line E. In the TTR gene, Lines A and B were mono-allelic for FSAD0037. In addition, genotype frequency was analysed for each SNP across the 7 lines. In all cases, there was a significant difference ($p<0.0001$) between the 7 lines used in the study.

Table 4.9: Single nucleotide polymorphisms allele frequencies in the IIp gene

Line	FSAD0014			FSAD0016			FSAD0024			FSAD0025			FSAD0026		
	n	Allele 1 (A)	Allele 2 (C)	*n*	Allele 1 (C)	Allele 2 (T)	*n*	Allele 1 (A)	Allele 2 (G)	*n*	Allele 1 (G)	Allele 2 (C)	*n*	Allele 1 (C)	Allele2 (T)
Line A	42	0.107	0.893	42	0.821	0.179	42	0.798	0.202	42	0.774	0.226	42	0.869	0.131
Line B	47	0.074	0.926	47	0.936	0.064	47	0.830	0.170	47	0.830	0.170	47	0.830	0.170
Line C	57	0.930	0.070	57	0.000	1.000	57	0.000	1.000	57	0.000	1.000	57	0.000	1.000
Line D	36	0.625	0.375	36	0.361	0.639	36	0.347	0.653	36	0.347	0.653	36	0.347	0.653
Line E	62	0.718	0.282	65	0.023	0.977	65	0.000	1.000	65	0.000	1.000	64	0.016	0.984
Line F	47	0.298	0.702	47	0.606	0.394	48	0.594	0.406	48	0.594	0.406	48	0.688	0.313
Line G	47	0.489	0.511	48	0.406	0.594	48	0.323	0.677	47	0.323	0.677	48	0.394	0.606

Table 4.10: Single nucleotide polymorphisms allele frequencies in the CRP gene

	FSAD0003			FSAD0006			FSAD0013			FSAD0020		
Line	*n*	Allele 1 (T)	Allele 2 (C)	*n*	Allele 1 (C)	Allele 2 (T)	*n*	Allele 1 (T)	Allele 2 (C)	*n*	Allele 1 (C)	Allele 2 (T)
Line A	43	0.905	0.095	42	0.952	0.048	42	0.095	0.905	18	0.722	0.278
Line B	47	0.266	0.734	46	0.380	0.620	47	0.734	0.266	23	0.478	0.522
Line C	58	0.948	0.052	58	1.000	0.000	58	0.052	0.948	36	0.708	0.292
Line D	36	0.681	0.319	36	0.861	0.139	35	0.329	0.671	20	0.775	0.225
Line E	64	0.930	0.070	64	1.000	0.000	64	0.070	0.930	29	0.862	0.138
Line F	48	0.531	0.469	48	0.938	0.063	48	0.479	0.521	27	0.796	0.204
Line G	48	0.875	0.125	48	0.938	0.063	48	0.135	0.865	29	0.638	0.362

Table 4.11: Single nucleotide polymorphisms allele frequencies in the Pig-MAP gene

Line	FSAD0027			FSAD0031			FSAD0034			FSAD0035			FSAD0036		
	n	Allele 1 (G)	Allele 2 (A)	*n*	Allele 1 (C)	Allele 2 (T)	*n*	Allele 1 (C)	Allele 2 (T)	*n*	Allele 1 (C)	Allele 2 (T)	*n*	Allele 1 (C)	Allele 2 (T)
Line A	41	0.561	0.439	41	0.451	0.549	42	0.143	0.857	42	0.226	0.774	31	0.581	0.419
Line B	47	0.394	0.606	47	0.670	0.330	47	0.149	0.851	47	0.574	0.426	40	0.725	0.275
Line C	58	0.638	0.362	57	0.368	0.632	58	0.009	0.991	58	0.362	0.638	49	0.469	0.531
Line D	36	0.597	0.403	36	0.375	0.625	36	0.056	0.944	36	0.333	0.667	28	0.536	0.464
Line E	64	0.133	0.867	64	0.930	0.070	64	0.000	1.000	64	0.445	0.555	50	0.920	0.080
Line F	48	0.354	0.646	48	0.688	0.313	48	0.427	0.573	48	0.677	0.323	43	0.721	0.279
Line G	48	0.271	0.729	48	0.760	0.240	48	0.573	0.427	48	0.667	0.333	22	0.821	0.179

Table 4.12: Single nucleotide polymorphisms allele frequencies in the TTR gene

	FSAD0037			FSAD0038			FSAD0039		
Line	***n***	**Allele 1 (G)**	**Allele 2 (T)**	***n***	**Allele 1 (A)**	**Allele 2 (T)**	***n***	**Allele 1 (C)**	**Allele 2 (A)**
Line A	42	0.000	1.000	42	0.512	0.488	42	0.452	0.548
Line B	47	0.000	1.000	47	0.298	0.702	47	0.479	0.521
Line C	58	0.397	0.603	57	0.605	0.395	58	0.793	0.207
Line D	36	0.139	0.861	36	0.486	0.514	36	0.653	0.347
Line E	64	0.320	0.680	64	0.641	0.359	62	0.790	0.210
Line F	48	0.188	0.813	48	0.375	0.625	48	0.625	0.375
Line G	48	0.302	0.696	48	0.323	0.677	48	0.698	0.302

4.3.4. Linkage Disequilibrium and Hardy-Weinberg Equilibrium

4.3.4.1. Haptoglobin Gene

For each SNP, Hardy-Weinberg equilibrium (HWE) tests were performed, none were in agreement with Hardy-Weinberg distribution and all were highly significanct ($p<0.001$) (Table 4.13). The 5 SNP were in high linkage disequilibrium (LD) with each other (Table 4.14). Specifically FSAD0024, FSAD0025 and FSAD0026 are all in complete LD.

4.3.4.2. C-reactive Protein Gene

For each SNP, HWE tests were performed, again, none of the SNP were in agreement with Hardy-Weinberg distribution and showed high significance ($p<0.001$) (Table 4.15). Three of the SNP were found in strong LD with each other, with FSAD0003 in complete LD with FSAD0020 (Table 4.16).

4.3.4.3. Pig-MAP Gene

For each SNP, HWE tests were performed, and 2 (FSAD0035 and FSAD0036) out of the 5 SNP were in agreement with Hardy-Weinberg distribution. Three SNP (FSAD0027, FSAD0031 and FSAD0034) deviated from HWE with moderate to high significance ($p<0.05$) (Table 4.17). The 5 SNP were in strong LD with each other (average D′ for each combination was 0.851), with FSAD0031 and FSAD0035 in complete LD (Table 4.18).

4.3.4.4. Transthyretin Gene

For each SNP, HWE tests were performed, and 1 (FSAD0038) out of the 3 SNP was in agreement with Hardy-Weinberg distribution. The remaining 2 SNP (FSAD0037 and FSAD0039) deviated from HWE with high significance ($p<0.05$) (Table 4.19). The 3 SNP were in weak LD with each other (average D' for each combination was 0.425) (Table 4.20).

Table 4.13: Hardy-Weinberg distribution for SNP within the Hp gene. Data taken from SAS 9.1 output.

Obs	Locus1	Locus2	NIndiv	Test	ChiSq	DF	ProbChi
1	FSAD0014	FSAD0014	338	HWE	62.972	1	2.0965E-15
2	FSAD0014	FSAD0016	338	LD	203.347	1	3.885E-46
3	FSAD0014	FSAD0024	338	LD	176.803	1	2.4181E-40
4	FSAD0014	FSAD0026	338	LD	174.402	1	8.0871E-40
5	FSAD0014	FSAD0025	336	LD	204.210	1	2.5188E-46
6	FSAD0016	FSAD0016	342	HWE	83.181	1	7.4877E-20
7	FSAD0016	FSAD0024	342	LD	291.608	1	2.2193E-65
8	FSAD0016	FSAD0026	342	LD	287.984	1	1.3672E-64
9	FSAD0016	FSAD0025	340	LD	250.411	1	2.1129E-56
10	FSAD0024	FSAD0024	343	HWE	70.482	1	4.6444E-17
11	FSAD0024	FSAD0026	343	LD	338.758	1	1.1894E-75
12	FSAD0024	FSAD0025	341	LD	294.264	1	5.8555E-66
13	FSAD0026	FSAD0026	343	HWE	69.952	1	6.0767E-17
14	FSAD0026	FSAD0025	341	LD	290.606	1	3.6685E-65
15	FSAD0025	FSAD0025	341	HWE	88.607	1	4.8165E-21

Table 4.14: Linkage Disequilibrium Measures for SNP within the Hp Gene. Data taken from SAS 9.1 output file.

Locus 1	Locus 2	Haplotype	Frequency	LD. Coeff.	Lewontin's D'
FSAD0014	FSAD0016	1-1	0.0125	-0.1912	-0.9388
FSAD0014	FSAD0016	1-2	0.4757	0.1912	0.9388
FSAD0014	FSAD0016	2-1	0.4047	0.1912	0.9388
FSAD0014	FSAD0016	2-2	0.1071	-0.1912	-0.9388
FSAD0014	FSAD0024	1-1	0.0095	-0.1754	-0.9486
FSAD0014	FSAD0024	1-2	0.4787	0.1754	0.9486
FSAD0014	FSAD0024	2-1	0.3692	0.1754	0.9486
FSAD0014	FSAD0024	2-2	0.1426	-0.1754	-0.9486
FSAD0014	FSAD0026	1-1	0.0095	-0.1739	-0.9481
FSAD0014	FSAD0026	1-2	0.4786	0.1739	0.9481
FSAD0014	FSAD0026	2-1	0.3662	0.1739	0.9481
FSAD0014	FSAD0026	2-2	0.1456	-0.1739	-0.9481
FSAD0014	FSAD0025	1-1	0.0094	-0.1919	-0.9533
FSAD0014	FSAD0025	1-2	0.4772	0.1919	0.9533
FSAD0014	FSAD0025	2-1	0.4043	0.1919	0.9533
FSAD0014	FSAD0025	2-2	0.1091	-0.1919	-0.9533
FSAD0016	FSAD0024	1-1	0.3743	0.2200	1.0000
FSAD0016	FSAD0024	1-2	0.0380	-0.2200	-1.0000
FSAD0016	FSAD0024	2-2	0.5877	0.2200	1.0000
FSAD0016	FSAD0026	1-1	0.3713	0.2200	1.0000
FSAD0016	FSAD0026	1-2	0.0409	-0.2182	-1.0000
FSAD0016	FSAD0026	2-2	0.5877	0.2182	1.0000
FSAD0016	FSAD0025	1-1	0.3774	0.2079	0.8634
FSAD0016	FSAD0025	1-2	0.0358	-0.2079	-0.8634
FSAD0016	FSAD0025	2-1	0.0329	-0.2079	-0.8634
FSAD0016	FSAD0025	2-2	0.5539	0.2079	0.8634
FSAD0024	FSAD0026	1-1	0.3732	0.2328	1.0000
FSAD0024	FSAD0026	1-2	0.0029	-0.2328	-1.0000
FSAD0024	FSAD0026	2-2	0.6239	0.2328	1.0000
FSAD0024	FSAD0025	1-1	0.3768	0.2216	1.0000
FSAD0024	FSAD0025	2-1	0.0352	-0.2216	-1.0000
FSAD0024	FSAD0025	2-2	0.5880	0.2216	1.0000
FSAD0026	FSAD0025	1-1	0.3739	0.2198	1.0000
FSAD0026	FSAD0025	2-1	0.0381	-0.2198	-1.0000
FSAD0026	FSAD0025	2-2	0.5880	0.2198	1.0000

Table 4.15: Hardy-Weinberg distribution for SNP within the CRP gene. Data taken from SAS 9.1 output.

Obs	Locus1	Locus2	NIndiv	Test	ChiSq	DF	ProbChi
1	FSAD0003	FSAD0003	343	HWE	22.319	1	0.000002
2	FSAD0003	FSAD0013	342	LD	315.936	1	0.000000
3	FSAD0003	FSAD0006	182	LD	3.335	1	0.067803
4	FSAD0003	FSAD0020	342	LD	142.805	1	0.000000
5	FSAD0013	FSAD0013	342	HWE	25.950	1	0.000000
6	FSAD0013	FSAD0006	182	LD	3.125	1	0.077090
7	FSAD0013	FSAD0020	341	LD	135.307	1	0.000000
8	FSAD0006	FSAD0006	182	HWE	43.424	1	0.000000
9	FSAD0006	FSAD0020	182	LD	6.229	1	0.012571
10	FSAD0020	FSAD0020	342	HWE	83.006	1	0.000000

Table 4.16: Linkage Disequilibrium Measures for SNP within the CRP gene. Data taken from SAS 9.1 output.

Locus1	Locus2	Haplotype	Frequency	LD Coeff	Lewontin's D'
FSAD0003	FSAD0013	1-1	0.0088	-0.1816	-0.9686
FSAD0003	FSAD0013	1-2	0.7397	0.1816	0.9686
FSAD0003	FSAD0013	2-1	0.2456	0.1816	0.9686
FSAD0003	FSAD0013	2-2	0.0059	-0.1816	-0.9686
FSAD0003	FSAD0006	1-1	0.5566	0.0268	0.1451
FSAD0003	FSAD0006	1-2	0.1852	-0.0268	-0.1451
FSAD0003	FSAD0006	2-1	0.1577	-0.0268	-0.1451
FSAD0003	FSAD0006	2-2	0.1005	0.0268	0.1451
FSAD0003	FSAD0020	1-1	0.7515	0.0912	1.0000
FSAD0003	FSAD0020	2-1	0.1272	-0.0912	-1.0000
FSAD0003	FSAD0020	2-2	0.1213	0.0912	1.0000
FSAD0013	FSAD0006	1-1	0.1660	-0.0263	-0.1365
FSAD0013	FSAD0006	1-2	0.1032	0.0263	0.1365
FSAD0013	FSAD0006	2-1	0.5482	0.0263	0.1365
FSAD0013	FSAD0006	2-2	0.1825	-0.0263	-0.1365
FSAD0013	FSAD0020	1-1	0.1321	-0.0894	-0.9827
FSAD0013	FSAD0020	1-2	0.1201	0.0894	0.9827
FSAD0013	FSAD0020	2-1	0.7462	0.0894	0.9827
FSAD0013	FSAD0020	2-2	0.0016	-0.0894	-0.9827
FSAD0006	FSAD0020	1-1	0.6518	0.0278	0.3076
FSAD0006	FSAD0020	1-2	0.0625	-0.0278	-0.3076
FSAD0006	FSAD0020	2-1	0.2218	-0.0278	-0.3076
FSAD0006	FSAD0020	2-2	0.0639	0.0278	0.3076

Table 4.17: Hardy-Weinberg distribution for SNP within the Pig-MAP gene. Data taken from SAS 9.1 output.

Obs	Locus1	Locus2	NIndiv	Test	ChiSq	DF	ProbChi
1	FSAD0027	FSAD0027	342	HWE	4.947	1	0.02614
2	FSAD0027	FSAD0031	340	LD	272.787	1	0.00000
3	FSAD0027	FSAD0035	342	LD	152.240	1	0.00000
4	FSAD0027	FSAD0034	342	LD	33.866	1	0.00000
5	FSAD0027	FSAD0036	282	LD	173.898	1	0.00000
6	FSAD0031	FSAD0031	341	HWE	10.985	1	0.00092
7	FSAD0031	FSAD0035	341	LD	183.221	1	0.00000
8	FSAD0031	FSAD0034	341	LD	43.562	1	0.00000
9	FSAD0031	FSAD0036	282	LD	211.912	1	0.00000
10	FSAD0035	FSAD0035	343	HWE	0.048	1	0.82620
11	FSAD0035	FSAD0034	343	LD	83.217	1	0.00000
12	FSAD0035	FSAD0036	283	LD	114.778	1	0.00000
13	FSAD0034	FSAD0034	343	HWE	16.197	1	0.00006
14	FSAD0034	FSAD0036	283	LD	27.622	1	0.00000
15	FSAD0036	FSAD0036	283	HWE	3.253	1	0.07131

Table 4.18: Linkage Disequilibrium Measures for SNP within the Pig-MAP gene. Data taken from SAS 9.1 output.

Locus1	Locus2	Haplotype	Frequency	LD Coeff	Lewontin's D'
FSAD0027	FSAD0031	1-1	0.0415	- 0.2128	-0.9588
FSAD0027	FSAD0031	1-2	0.3644	0.2128	0.9588
FSAD0027	FSAD0031	2-1	0.5850	0.2128	0.9588
FSAD0027	FSAD0031	2-2	0.0091	-0.2128	-0.9588
FSAD0027	FSAD0035	1-1	0.0289	-0.1636	-0.8500
FSAD0027	FSAD0035	1-2	0.3761	0.1636	0.8500
FSAD0027	FSAD0035	2-1	0.4463	0.1636	0.8500
FSAD0027	FSAD0035	2-2	0.1487	-0.1636	-0.8500
FSAD0027	FSAD0034	1-1	0.0151	-0.0601	-0.7988
FSAD0027	FSAD0034	1-2	0.3898	0.0601	0.7988
FSAD0027	FSAD0034	2-1	0.1705	0.0601	0.7988
FSAD0027	FSAD0034	2-2	0.4245	-0.0601	-0.7988
FSAD0027	FSAD0036	1-1	0.0915	-0.1759	-0.9375
FSAD0027	FSAD0036	1-2	0.2932	0.1759	0.9375
FSAD0027	FSAD0036	2-1	0.6035	0.1759	0.9375
FSAD0027	FSAD0036	2-2	0.0117	-0.1759	-0.9375
FSAD0031	FSAD0035	1-1	0.4736	0.1771	1.0000
FSAD0031	FSAD0035	1-2	0.1525	-0.1771	-1.0000
FSAD0031	FSAD0035	2-2	0.3739	0.1771	1.0000
FSAD0031	FSAD0034	1-1	0.1839	0.0673	0.9669
FSAD0031	FSAD0034	1-2	0.4422	-0.0673	-0.9669
FSAD0031	FSAD0034	2-1	0.0023	-0.0673	-0.9669
FSAD0031	FSAD0034	2-2	0.3716	0.0673	0.9669
FSAD0031	FSAD0036	1-1	0.6397	0.1910	0.9623
FSAD0031	FSAD0036	1-2	0.0075	-0.1910	-0.9623
FSAD0031	FSAD0036	2-1	0.0536	-0.1910	-0.9623
FSAD0031	FSAD0036	2-2	0.2992	0.1910	0.9623
FSAD0035	FSAD0034	1-1	0.1832	0.0955	0.9805
FSAD0035	FSAD0034	1-2	0.2905	-0.0955	-0.9805
FSAD0035	FSAD0034	2-1	0.0019	-0.0955	-0.9805
FSAD0035	FSAD0034	2-2	0.5243	0.0955	0.9805
FSAD0035	FSAD0036	1-1	0.4902	0.1467	0.9701
FSAD0035	FSAD0036	1-2	0.0045	-0.1467	-0.9701
FSAD0035	FSAD0036	2-1	0.2042	-0.1467	-0.9701
FSAD0035	FSAD0036	2-2	0.3011	0.1467	0.9701
FSAD0034	FSAD0036	1-1	0.1947	0.0573	0.9480
FSAD0034	FSAD0036	1-2	0.0031	-0.0573	-0.9480
FSAD0034	FSAD0036	2-1	0.4996	-0.0573	-0.9480
FSAD0034	FSAD0036	2-2	0.3025	0.0573	0.9480

Table 4.19: Hardy-Weinberg distribution for SNP within the TTR gene. Data taken from SAS 9.1 output.

Obs	Locus1	Locus2	NIndiv	Test	ChiSq	DF	ProbChi
1	FSAD0038	FSAD0038	342	HWE	0.0751	1	0.78410
2	FSAD0038	FSAD0037	342	LD	81.3593	1	0.00000
3	FSAD0038	FSAD0039	340	LD	18.1309	1	0.00002
4	FSAD0037	FSAD0037	343	HWE	5.0263	1	0.02496
5	FSAD0037	FSAD0039	341	LD	12.6487	1	0.00038
6	FSAD0039	FSAD0039	341	HWE	26.6080	1	0.00000

Table 4.20: Linkage Disequilibrium Measures for SNP within the TTR gene. Data taken from SAS 9.1 output.

Locus1	Locus2	Haplotype	Frequency	LD Coeff	Lewontin's D'
FSAD0038	FSAD0039	2-1	0.2925	-0.0548	-0.3381
FSAD0038	FSAD0039	2-2	0.2369	0.0548	0.3381
FSAD0037	FSAD0039	1-1	0.1757	0.0374	0.5134
FSAD0037	FSAD0039	1-2	0.0354	-0.0374	-0.5134
FSAD0037	FSAD0039	2-1	0.4797	-0.0374	-0.5134
FSAD0037	FSAD0039	2-2	0.3092	0.0374	0.5134

4.4.Discussion

Previous studies have sequenced APP genes and identified a small number of SNP within them. However, these studies have been performed on small numbers of animals and not always on the same breed or commercial lines, making it difficult to compare the data (Chomdej *et al.*, 2004, Ponsuksili *et al.*, 2002, Baskin *et al.*, 1998, Archibald *et al.*, 1996). The aim of this study was to identify SNP within the APP genes of a commercial boar population consisting of 7 lines of animals and to select SNP from each gene to be genotyped across the population. The data from this study is to be used in conjunction with the phenotypic data from Chapter 3 in order to determine associations between genotype and phenotype (Results in Chapter 5). Any associations that are found may prove useful if APP are to be used as biomarkers of herd health.

Sequencing a DNA pool consisting of the lines of interest identified 71 SNP, of which 17 were selected to be genotyped across the boar population. Selection criteria were based upon ease and method of genotyping and the time constraints of this study. Genotyping was carried out using one of three methods; PCR-RFLP, Pyrosequencing® and SNaPshot®. Each of these methods has advantages and disadvantages as discussed, but the choice of methods allowed each SNP to be genotyped accurately and allowed for a high volume of samples to be analysed quickly and efficiently. However, both PCR-RFLP and Pyrosequencing® appeared to be the more robust and 'user-friendly' of the three methods; being simple to perform and resulting in relatively low numbers of retests, hence making them more efficient overall.

Of the 17 SNP genotyped for across the population, most were found in intronic regions of sequence or were identified using BES data. Only one was identified within an exonic region (FSAD0038), and it was a silent mutation. Initially, it was thought that only non-synonymous polymorphisms found within coding regions of the genome could be of any value, however, it is now thought that polymorphisms in the non-coding regions of genome may contribute to gene expression, particularly where they are found in regulatory regions (Knight, 2003). If this is indeed the case, then it is possible that the SNP genotyped in this study may yet prove to have associations with APP concentrations in serum. Additionally, non-coding polymorphisms may be in LD with undiscovered coding SNP making them useful as markers if an association occurs.

Only one of the 17 SNP genotyped in this study had previously been described. This was the exonic polymorphism, FSAD0038, which was found in the TTR gene (Archibald *et al.*, 1996). Interestingly, this SNP was only reported in one study, a previous sequencing study failed to identify it. This lends weight to the belief that it can be difficult to compare data from different studies (Duan *et al.*, 1995).

Analysis of the individual SNP revealed that the majority of them were not in Hardy-Weinberg distribution. This is not surprising considering that HWE is based upon a large randomly mated population with no selection, mutation or migration occurring. In contrast, this study used a small commercial pig population under selection for desired characteristics and it is likely that gene pooling has occurred over time. Additionally, the majority of the SNP were found in strong LD with each other within genes. This may be an

effect of selective breeding but more likely due to the small distances between the SNP which decreases the chance of recombination occurring.

Generally, where a large number of SNP are found in strong or complete LD with each other, they can be used as markers for each other and only one marker from the group would be selected for genotyping. In this case, time was limited and there was not the opportunity to genotype more SNP. However, should the data gained from this study prove to be of significance, this point can be re-addressed with the SNP targeted in any future studies being evaluated for LD before genotyping the population and selected SNP being in lower LD with each other.

The allele frequencies of the tested SNP varied significantly across lines. Each line has been bred to meet particular criteria for production traits and may be a composite of several breeds. It would appear that the APP genes have either been affected by this breeding or that different breeds express different polymorphisms. In particular, several lines are monoallelic for certain SNP, for example Line A (Landrace) and Line B (LW) are monoallelic for FSAD0037 (TTR), Line C (Duroc) is monoallelic for FSAD0006 (CRP) and FSAD0016, FSAD0024, FSAD0025, FSAD0026 (all Hp). Line E (based on the Hampshire breed) is monoallelic for FSAD0024 and FSAD0025 (both Hp) and FSAD0034 (Pig-MAP). The difference in allele frequencies between lines may prove to be of significance in later studies if there is an association with APP concentration, and it should be noted that it is pure bred lines that are expressing this and not the lines based on breed

crosses (as breed composites, these should be more polymorphic than a line based on a pure breed).

This study identified novel SNP within the APP genes and has genotyped 17 of these across a boar population. Statistical analysis of these results has shown that there are significant line differences between the SNP. This data will be further utilized in a genotype-phenotype association study (Chapter 5). Additionally, the results indicate that genotyping of the remaining SNP may prove useful if the resources are available, particularly SNP which are found to be in low LD with each other.

Chapter V

5. Association between SNP in APP Genes and Baseline APP Serum Concentration

5.1. Genetic Association Studies

The aim of genetic association studies is to identify associations between genetic polymorphisms, for example SNP, and the trait of interest, in this case, the baseline concentration of APP. There are two ways to identify associations, either look for a direct association or an indirect association. Direct associations use polymorphisms which are candidate causal variants, for example, a nonsynonymous SNP. However, non-coding polymorphisms can also be considered candidate causal variants as they may cause variation in gene expression and regulation (Cordell and Clayton, 2005). Indirect association studies use polymorphisms which are in non-random association or linkage disequilibrium (LD) with a causal variant. However, indirect association studies are more difficult to analyse and less powerful than direct associations. In addition, they are a weaker association compared to a direct one and require a number of polymorphism to be identified (Cordell and Clayton, 2005, Wang and Todd, 2003).

5.1.1. Linkage Association

Linkage is the tendency for the alleles of genes to be passed together and in the same sequence from one generation to the next making it possible to follow transmission of the alleles from one generation to the next. This is due to the alleles being situated close together on the gene which makes it unlikely that a recombinant or cross-over

event will occur during meiosis. Linkage analysis involves finding a model to explain the inheritance pattern of phenotypes and genotypes observed in a population. They are useful in instances where traits do not show normal Mendelian inheritance patterns and linked genes can be used as genetic markers where the exact location of the causal gene is unknown (Nicholas, 2005, Lander and Schork, 1994).

5.1.2. Statistical Analysis

Two relatively simple but appropriate statistical methods for determining association between genotype and a quantitative phenotype are linear regression and analysis of variance (ANOVA). Both require the data to be normally distributed, and in some cases transformation, such as a log transformation, may be required (Balding, 2006).

5.1.2.1. Linear Regression

Linear regression describes the relationship between X and Y and can determine how much of the variation in Y can be explained by the relationship with X. This can be shown by the equation;

$$Y_i = \beta_0 + \beta_1 x_i + \epsilon_i \qquad i = 1, 2, 3, \ldots n$$

Where:

Y_i = value of Y for the i^{th} observation

β_0 = the intercept i.e. the value of Y when x = 0

β_1 = the slope i.e. measures the change in Y per unit change in X

ϵ_i = the random error associated with the i^{th} observation

(Quinn and Keough, 2002)

5.1.2.2. Analysis of Variance

In order to compare groups of samples, analysis of variance (ANOVA) assumes normal distribution of the residuals. Whereas a *t*-test compares 2 groups of samples, ANOVA compares 3 or more groups to assert whether the variability of the group means differs more than would be expected by chance. If there is a significant difference, then the difference between individual groups can be tested using a series of *t*-tests comparing the residual variance (Altman and Bland, 1996).

In statistical software programs, for example, SAS 9.1, an ANOVA procedure can be expressed as a linear model in which the model is used to predict the response for each observation. The difference between the actual and predicted response is termed the residual error and most models minimise the sum of squares of residual errors; this is known as least squares regression (SAS, 2004).

5.1.2.3. General Linear Model

An extension of these two methods is the general linear model (GLM). In the GLM, it is assumed that there is a normal distribution of the values in the population (again transformations can be used), that there is a linear relationship between the 2 variables and that the variance is the same for all responses. It is based upon the same equation as the linear model but with more variables;

$$Y_i = \beta_o x_{oi} + \beta_1 x_{1i} + \ldots \beta_k x_{ki} + \epsilon_i \qquad i = 1, 2, 3, \ldots n$$

Where:

Y_i = is the response

β_k = are unknown parameters to be estimated

x_1 = are design variables

ϵ_i = the random errors associated with the observations

The use of GLM allows the testing of linear combinations of the X variables, linear combinations of the Y variables and linear combinations of both the X and Y variables. This enables the analysis of a wide range of variables using a variety of tests including ANOVA, regression, analysis of covariance and multivariate analysis of variance (Dobson, 2002).

One of the most important features of a GLM is that of the model used. The model must be correctly specified otherwise the estimates of the parameters will be inaccurate and the resulting equation will not describe the data properly (SAS, 2004).

The aim of this study was to identify associations between APP baseline concentrations in a boar population from a high health herd and SNP genotyped in the APP genes (as described in Chapter 4).

5.2. Materials and Methods

5.2.1. Boars

A sub-population (n = 345) of the high health pig herd described in Chapter 3 was used in this study as only boars in this sub-population had both phenotypic (serum samples) and genetic (tail sample) material available for testing. The boar have been described in sections 3.21 to 3.24 and were from 7 pig lines comprising pure breed and synthetic lines as previously described.

5.2.2. Genotyping

Genotyping was carried out as described in Chapter 4. In short, SNP were identified from pooled DNA and 17 were chosen to be genotyped across the boar population using PCR-RFLP, Pyrosequencing® or SNaPshot®. Of the 17 SNP genotyped, 5 were located in the Hp gene, 4 in the CRP gene, 5 in the Pig-MAP gene and 3 in the TTR gene.

Genotypic data was verified by 2 people as per company protocol and was analysed for allele frequencies, HWE and LD as described in section 4.2.6.

5.2.3. Acute Phase Protein Determination

The four APP; Hp, CRP, Pig-MAP and TTR were measured in serum samples from the genotyped boars in duplicate as described in sections 3.2.5. Internal control samples were included with each assay run to ensure assay accuracy and validity. Where

possible, animals were bled twice to enable an average baseline level to be used for analysis.

5.2.4. Statistical Analysis

All analyses were carried out using SAS 9.1 statistical software (SAS Institute, Cary, NC, USA). Data was log transformed prior to analysis in order to render skewed distributions as close to normal distribution as possible. Associations of the genotypes with the APP concentrations were evaluated using a general linear model (PROC GLM) with line and SNP genotype as fixed effects. This can be shown as;

$$Y_{ij} = \mu + L_i + G_j + \epsilon_{ij}$$

where Y_{ij} is the trait measured in the *n*th animal of the *i*th line and *j*th genotype; μ is the overall mean for the trait. L_i is the fixed effect of the *i*th line and G_j is the fixed effect of the *j*th genotype.

5.3. Results

5.3.1. Genotyping

Allele frequencies, HWE and LD results have been described previously in sections 4.33 and 4.34.

5.3.2. Serum Concentrations of APP in a High Health Population

Serum concentrations of Hp, CRP, Pig-MAP and TTR across a population of boar from a high health herd are shown in Figures 5.1 – 5.4, respectively. The mean Hp, CRP and Pig-MAP concentrations were 0.34 g/L (range = 0 – 2.65 g/L), 29.9 mg/L (range = 0.9 – 485.2 mg/L) and 1.22 mg/ml (range = 0.11 – 9.63 mg/ml) respectively. The mean TTR concentration was 331.3 μg/ml (range = 119.2 – 820.3 μg/ml). Summary values are shown in Table 5.1.

5.3.3. Serum Concentrations of APP across Pig Lines

Serum concentrations of Hp, CRP, Pig-MAP and TTR in all lines are shown in Figures 5.5 – 5.9, respectively. Summary values are given in Tables 5.2 – 5.6.

Table 5.1: Summary values of Hp, CRP, Pig-MAP and TTR concentration in a high health boar population (n = 345)

	Hp (g/L)	CRP (mg/L)	Pig-MAP (mg/ml)	TTR (µg/ml)
n	343	345	342	343
Min.	0	0.9	0.11	119.2
Max.	2.65	485.2	9.63	820.3
Median	0.25	12.7	1.09	311.7
Mean	0.34	29.9	1.22	330.8
SEM	0.02	3.06	0.04	6.11
SD	0.34	56.76	0.79	113.07

Abbreviations: n: no. of pigs. SEM: standard error of the mean. SD: standard deviation.

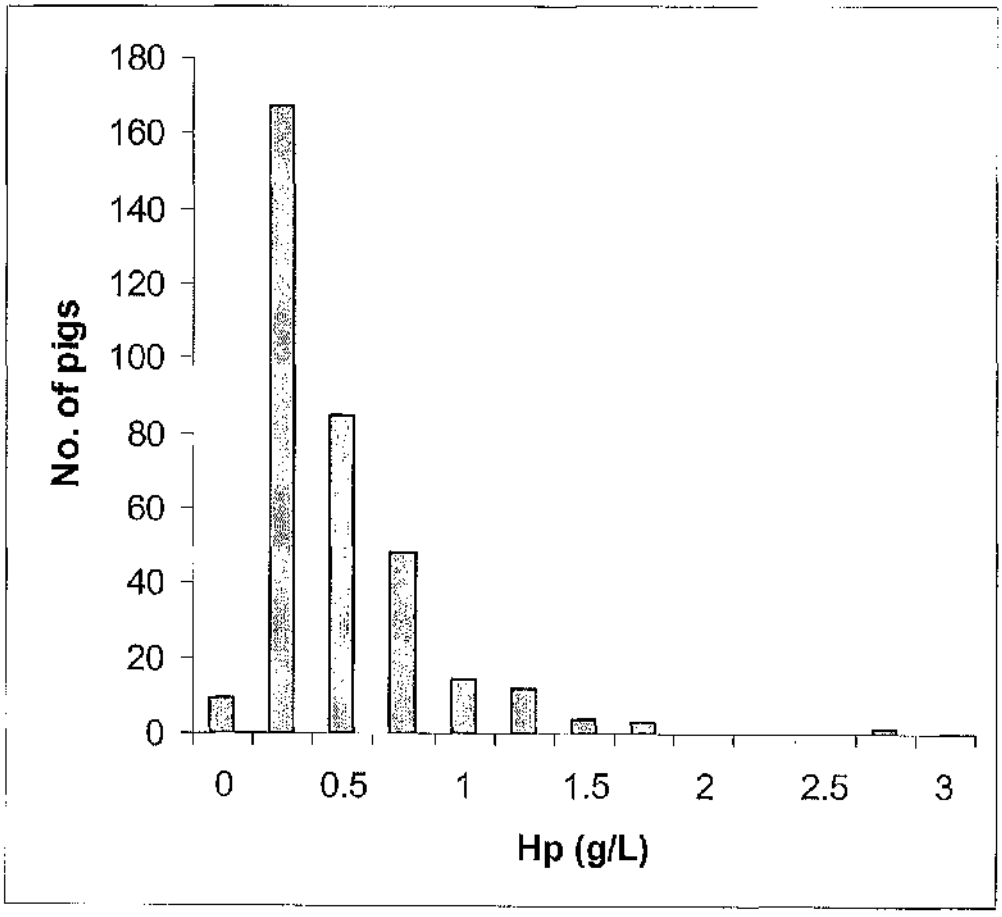


Figure 5.1: Serum concentrations of Hp (g/L) across a high health boar population (n = 343).

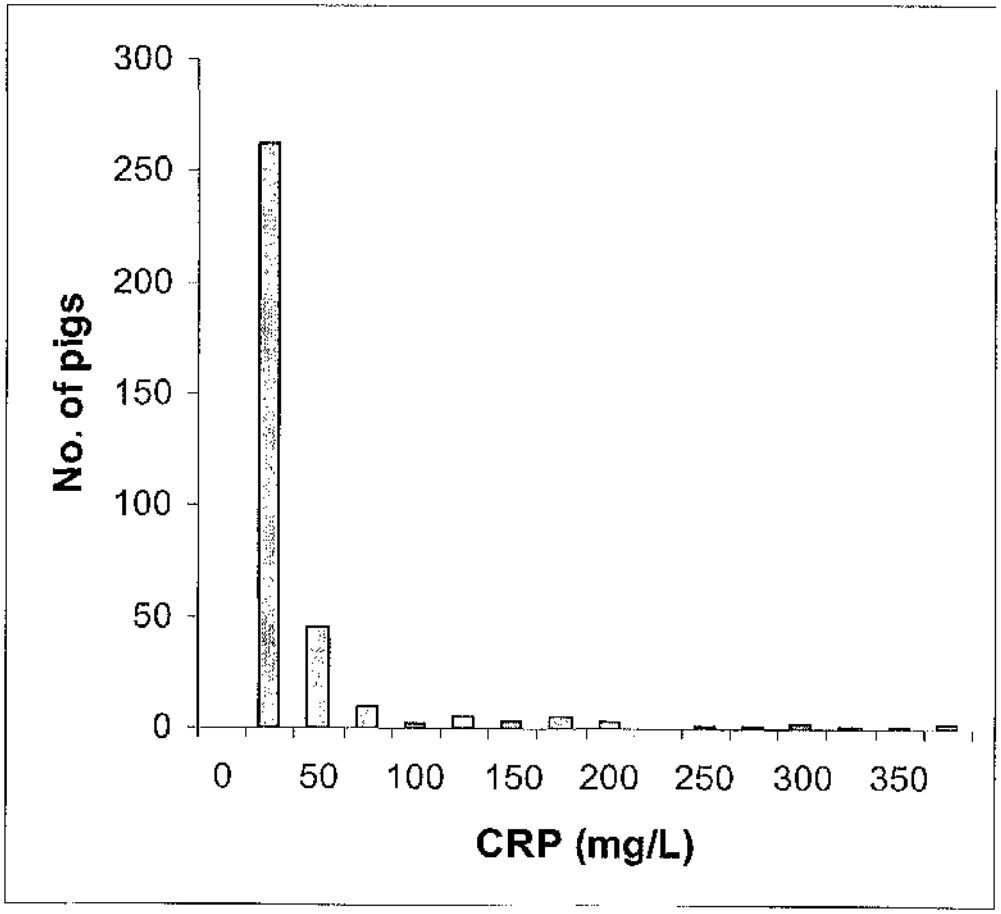


Figure 5.2: Serum concentrations of CRP (mg/L) across a high health boar population (n = 345).

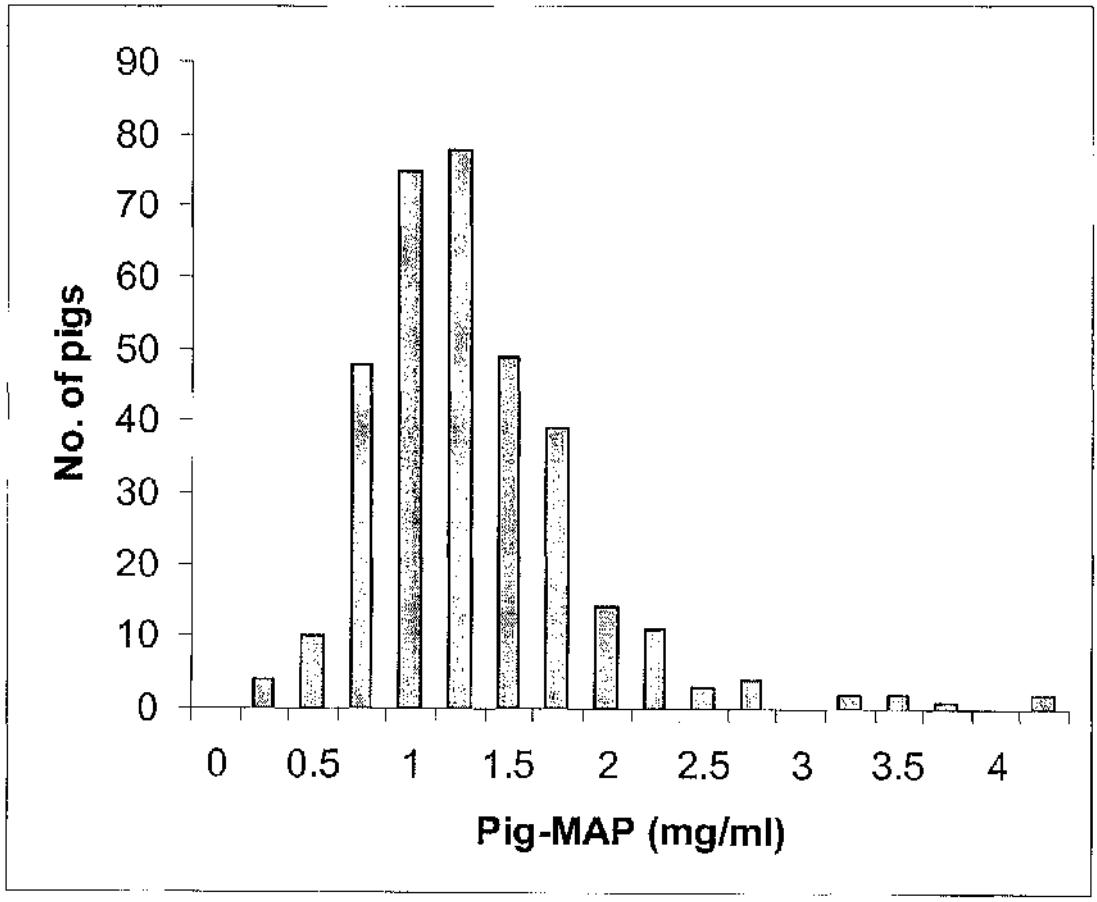


Figure 5.3: Serum concentrations of Pig-MAP (mg/ml) across a high health boar population (n = 342).

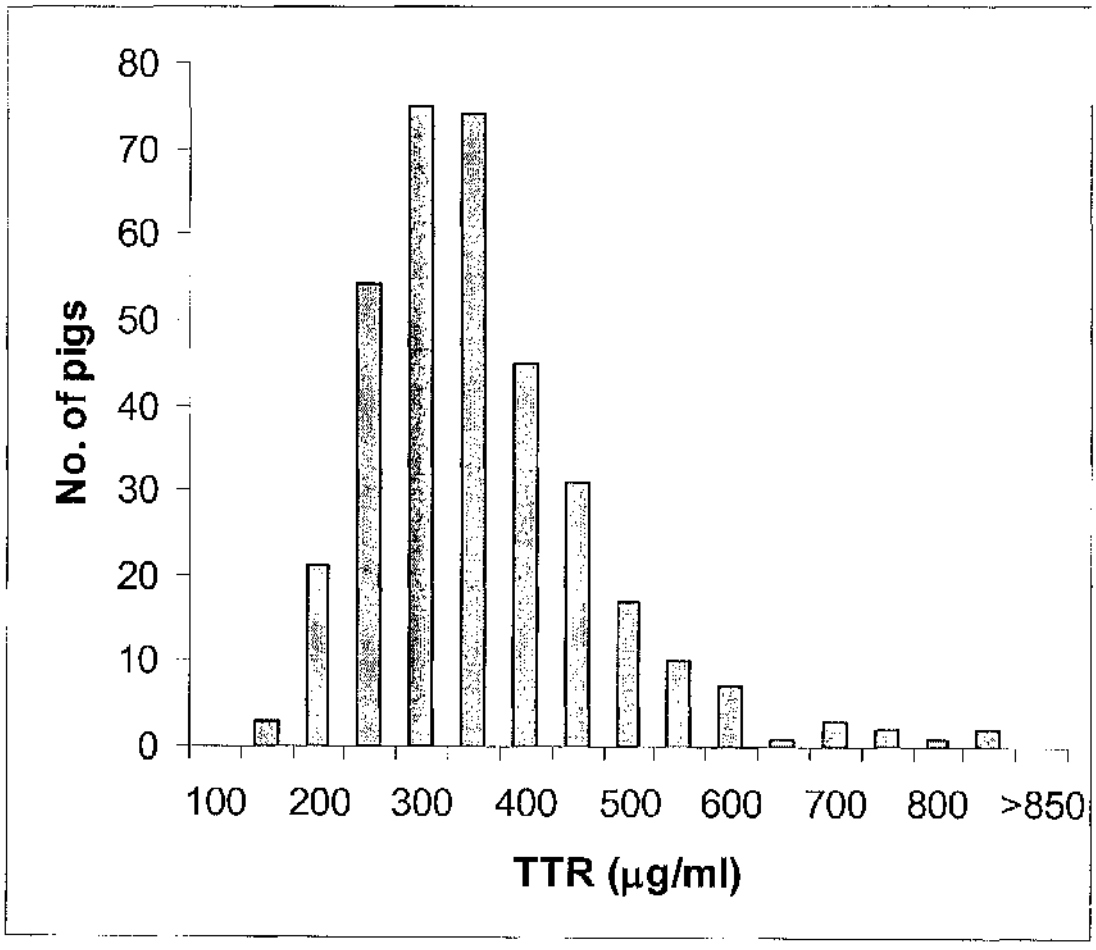


Figure 5.4: Serum concentrations of TTR (µg/ml) across a high health boar population (n = 343).

Table 5.2: Summary values of Hp (g/L) across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	42	47	57	36	65	48	48
Min.	0.01	0.00	0.00	0.00	0.00	0.00	0.02
Max	0.94	1.20	1.55	0.77	2.65	1.54	1.16
Median	0.28	0.29	0.22	0.15	0.26	0.23	0.30
Mean	0.31	0.39	0.33	0.21	0.38	0.41	0.35
SEM	0.03	0.04	0.04	0.03	0.06	0.07	0.04
SD	0.21	0.29	0.29	0.20	0.45	0.45	0.31

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation.

Table 5.3: Summary values of CRP (mg/L) across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	42	47	58	37	65	48	48
Min.	3.6	2.4	2.4	3.9	0.9	3.4	3.6
Max	269.5	191.1	238.7	63.9	378.1	485.2	183.0
Median	8.5	10.9	14.9	13.5	12.1	14.4	15.7
Mean	17.5	22.2	24.8	17.8	39.9	45.5	35.0
SEM	6.31	5.39	4.43	2.22	9.60	13.02	6.80
SD	40.86	36.94	33.74	13.48	77.39	90.20	47.11

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation.

Table 5.4: Summary values of Pig-MAP (mg/ml) across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	42	47	58	36	63	48	48
Min.	0.28	0.35	0.29	0.48	0.11	0.36	0.13
Max	2.07	3.64	2.74	2.02	9.63	3.13	3.33
Median	1.00	1.03	1.19	1.15	1.33	1.03	1.01
Mean	1.00	1.12	1.26	1.20	1.65	1.10	1.05
SEM	0.06	0.08	0.07	0.06	0.19	0.07	0.07
SD	0.36	0.52	0.54	0.37	1.48	0.48	0.51

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation.

Table 5.5: Summary values of TTR (μg/ml) across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	42	47	58	36	64	48	48
Min.	172.0	173.4	173.6	162.0	119.2	170.7	138.3
Max	459.2	801.8	571.4	595.9	820.3	529.3	457.8
Median	284.6	352.4	301.4	317.8	366.9	285.4	262.3
Mean	297.1	381.2	318.4	335.6	387.5	307.2	270.4
SEM	12.80	21.05	11.28	19.03	16.80	11.27	10.44
SD	82.94	144.32	85.92	114.16	134.43	78.08	72.35

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation.

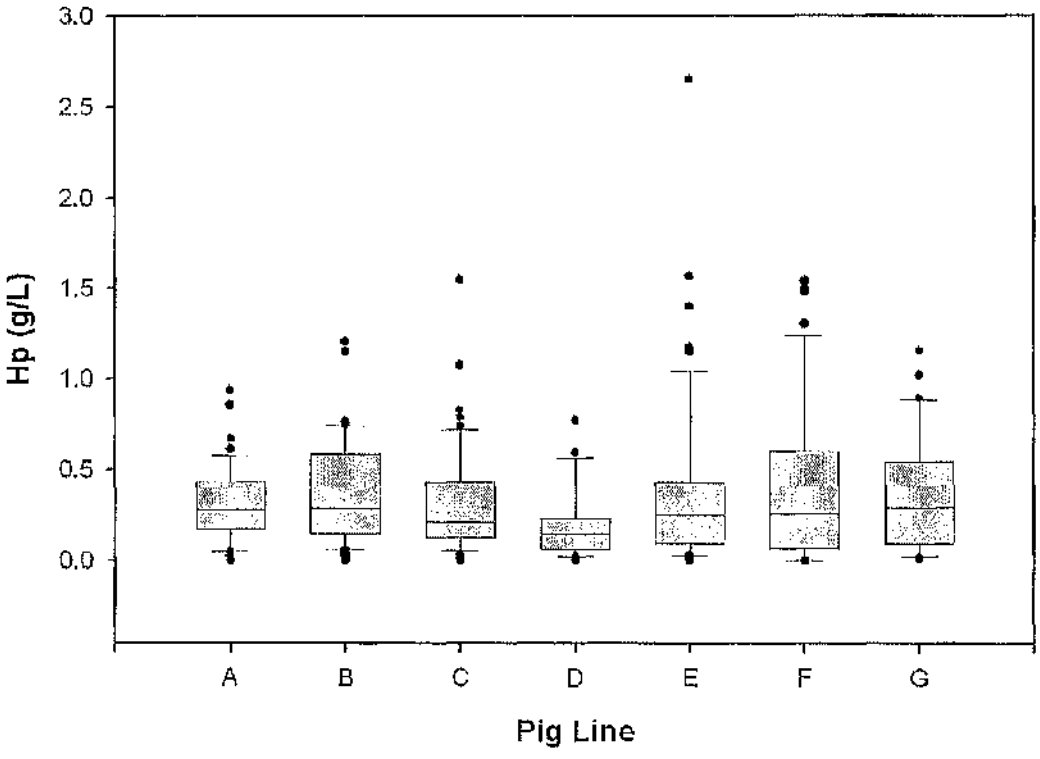


Figure 5.5: Hp concentration (g/L) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

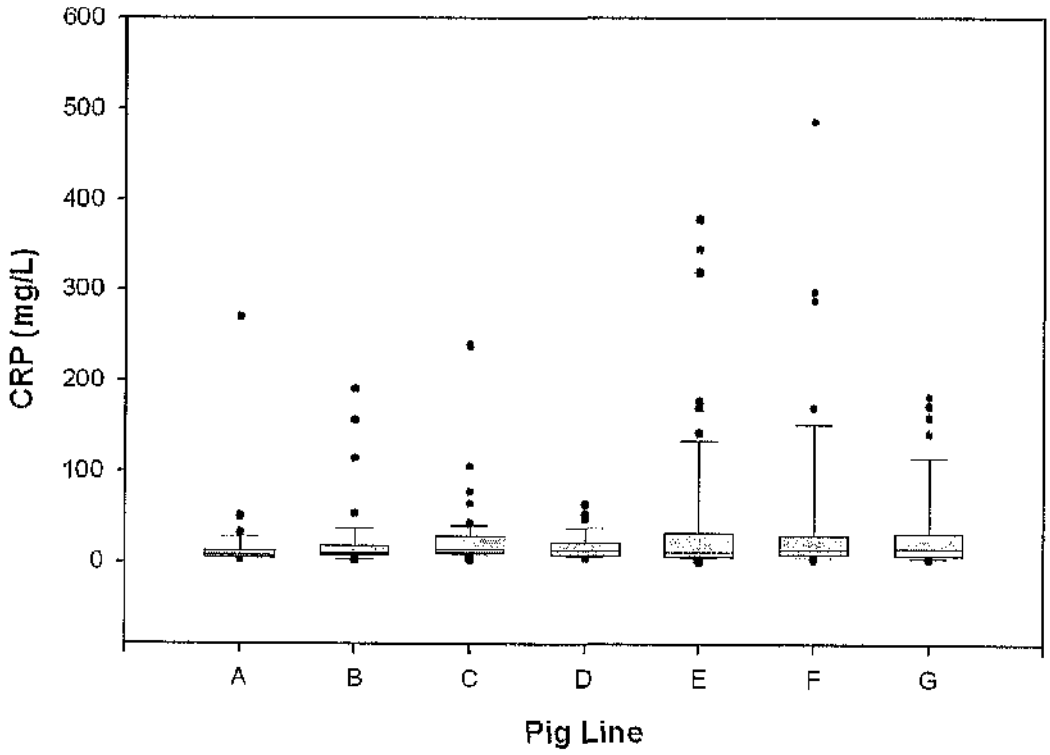


Figure 5.6: CRP concentration (mg/L) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

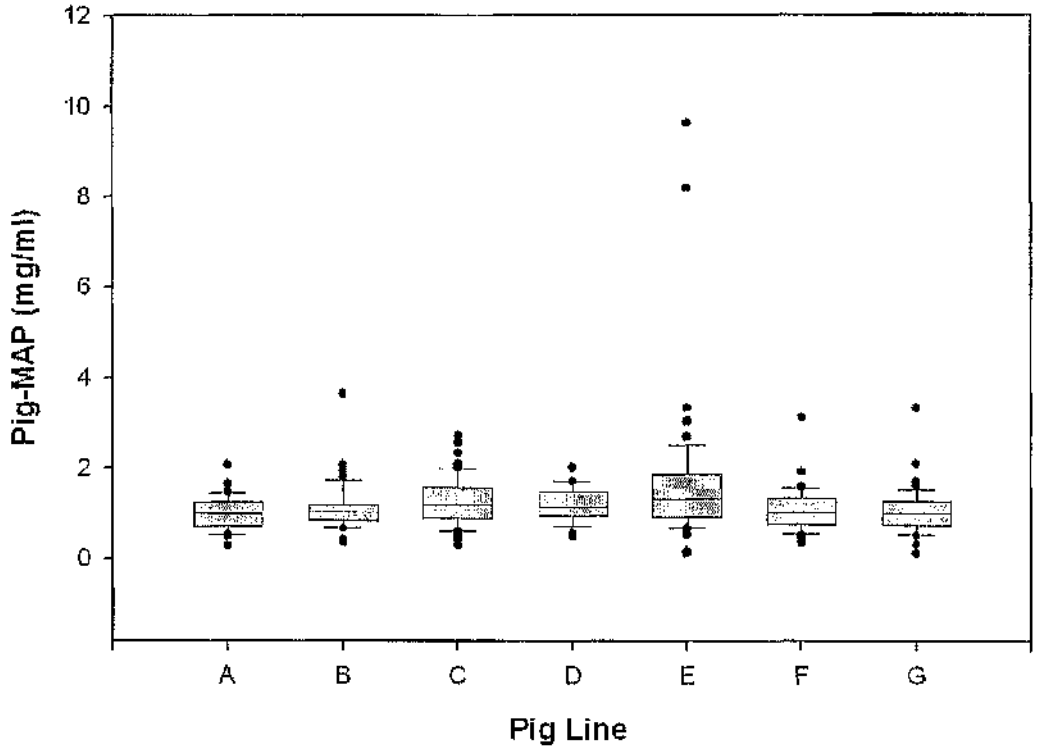


Figure 5.7: Pig-MAP concentration (mg/ml) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

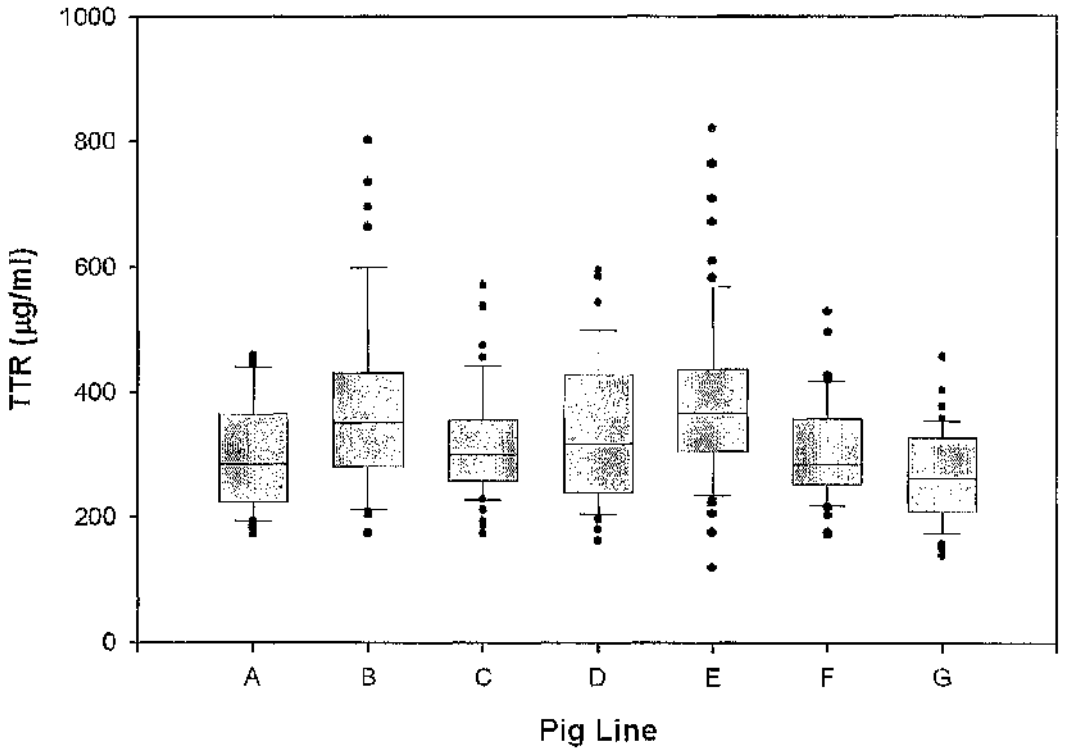


Figure 5.8: TTR concentrations (µg/ml) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

5.3.4. Association of Genotype with APP Concentration

5.3.4.1. Haptoglobin

There was a significant association between FSAD0016, FSAD0024, and FSAD0025 and the serum concentration of Hp, however there was no association found with FSAD0014 or FSAD0026 as shown in Table 5.6. There was no association found between line and Hp serum concentration.

5.3.4.2. CRP

There was no association found between genotype and the serum concentration of CRP. There were significant associations between line and CRP concentration as shown in Table 5.7.

5.3.4.3. Pig-MAP

There was no association found between genotype and the serum concentration of Pig-MAP. There were significant associations between line and Pig-MAP concentration as shown in Table 5.8.

5.3.4.4. TTR

There was no significant association found between genotype and the serum concentration of TTR. FSAD0039 had an association with baseline TTR concentrations approaching significance at p=0.07. There were significant associations between line and TTR concentration as shown in Table 5.9.

Table 5.6: Association of pig line and genotype in the Hp gene with Hp serum concentration.

	FSAD0014	FSAD0016	FSAD0024	FSAD0025	FSAD0026
Line	0.6503	0.2811	0.1087	0.1316	0.3122
Genotype	0.3908	0.0093**	0.0051**	0.0094**	0.1412

**$p < 0.01$

Table 5.7: Association of pig line and genotype in the CRP gene with CRP serum concentration

	FSAD0003	FSAD0006	FSAD0013	FSAD0020
Line	0.0157*	0.0427*	0.0169*	0.5251
Genotype	0.8908	0.2376	0.9528	0.6984

*$p < 0.05$

Table 5.8: Association of pig line and genotype in the Pig-MAP gene with Pig-MAP serum concentration

	FSAD0027	FSAD0031	FSAD0034	FSAD0035	FSAD0036
Line	0.0280*	0.0244*	0.0834	0.0820	0.0030**
Genotype	0.3624	0.2975	0.2118	0.4019	0.2986

*$p < 0.05$, **$p < 0.01$

Table 5.9: Association of pig line and genotype in the TTR gene with TTR serum concentration

	FSAD0038	FSAD0037	FSAD0039
Line	<0.0001***	<0.0001***	<0.0001***
Genotype	0.7761	0.8704	0.0708

***p < 0.001

5.3.5. Effect of Hp SNP Genotypes on Hp Serum Concentration

In Table 5.10, the least mean squares (LSMEAN) of the mean Hp concentration are given for the genotypes of FSAD0014, FSAD0016, FSAD0024, FSAD0025 and FSAD0026. A significant difference was obtained for LSMEAN between the genotypes of these SNP as indicated. The untransformed data is shown in Figure 5.9.

Table 5.10: Least squares means of mean Hp concentration by Hp SNP genotype

SNP	Genotype	LSMEAN
FSAD0014	AA	0.185
	AC	0.189
	CC	0.222
FSAD0016	CC	0.262*ab*
	CT	0.193*a*
	TT	0.153*b*
FSAD0024	AA	0.251*c*
	AG	0.233*d*
	GG	0.146*cd*
FSAD0025	GG	0.246*e*
	GC	0.233*f*
	CC	0.150*ef*
FSAD0026	CC	0.231
	CT	0.217
	TT	0.166

Identical script indicates significant difference ($p<0.05$) between genotypes

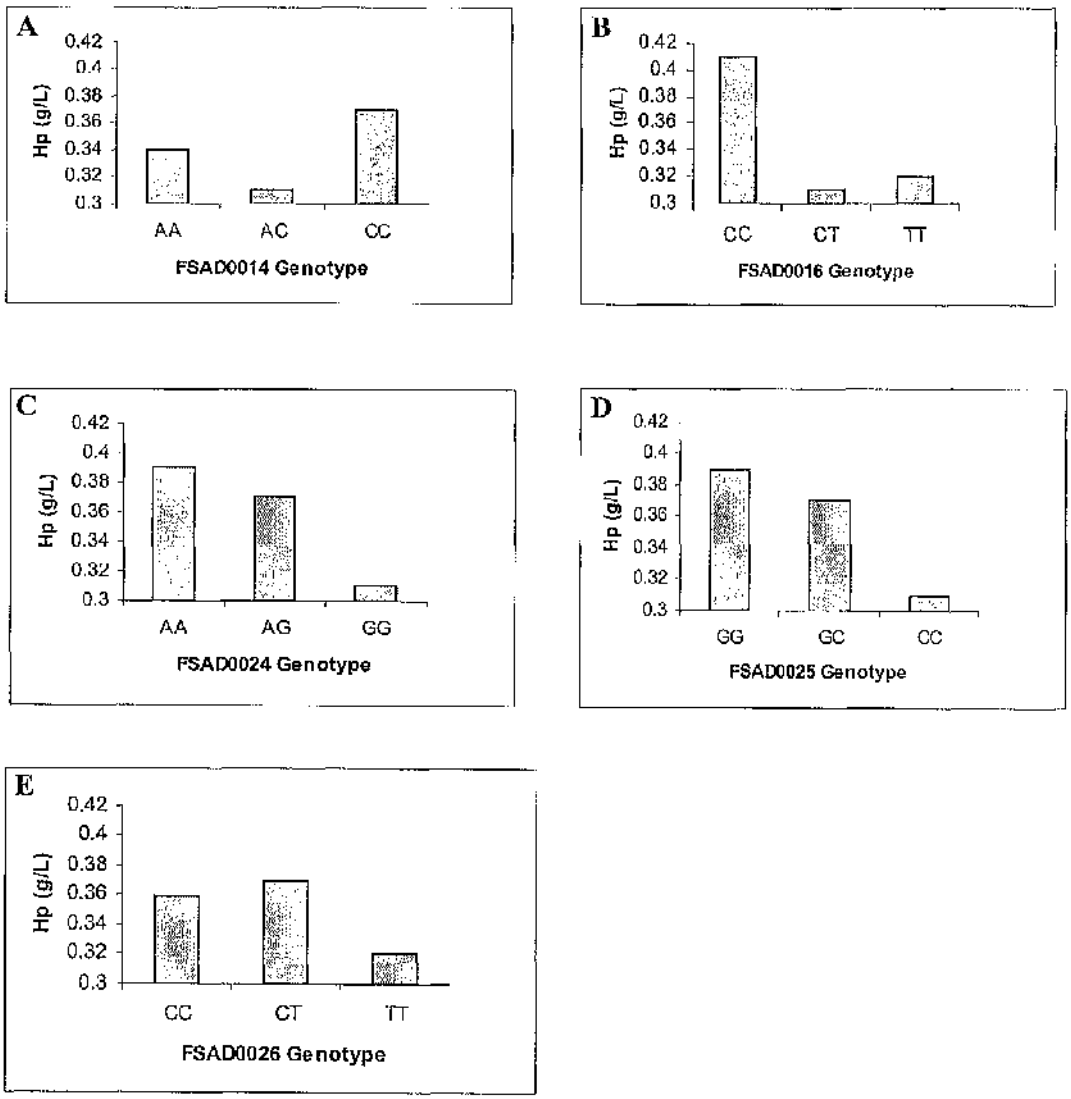


Figure 5.9: Mean Hp (g/L) depending on genotype of SNP in Hp gene. Untransformed data shown for A: FSAD0014, B: FSAD0016, C: FSAD0024, D: FSAD0025, E: FSAD0026.

5.4. Discussion

The aim of this study was to identify associations between SNP in the APP genes and baseline serum concentrations of the respective proteins. Prior to this study, similar work has only been carried out in humans where significant associations have been reported between SNP and both CRP and Hp serum concentrations (Wang *et al.*, 2006, Suk *et al.*, 2005, Teye *et al.*, 2006).

The boars used in this study were a sub-population of the herd examined in Chapter 3. This herd showed significant differences in CRP, Pig-MAP and TTR concentrations between lines, however no differences were apparent in Hp concentrations. As previously, the APP concentrations in the sub-population were shown to be skewed due to overall low APP concentrations. This can be considered normal for a high health herd and is commonly exhibited. The mean APP concentrations were within reported 'normal' ranges for boar apart from Pig-MAP (discussed previously in Chapter 3) and there were few outliers, indicating that there were little or no external influences (for example, sub-clinical disease or trauma) on the protein concentrations.

The association analysis revealed that there was a significant association between FSAD0016, FSAD0024 and FSAD0025 and Hp serum concentration and identified the genotypes responsible for lower Hp concentrations in FSAD0016 (allele T) and higher Hp concentrations in FSAD0024 (allele A) and FSAD0025 (allele G). However, there are no associations found between line and Hp concentration. There were no significant associations found between any other genotypes and serum protein concentration, however,

an association approaching significance was found between FSAD0039 and TTR concentrations, with the C allele associated with higher baseline concentrations. Associations between line and protein concentration were reported for FSAD0003, FSAD0006 and FSAD0013 (CRP), FSAD0027, FSAD0031 and FSAD0030 (Pig-MAP) and for all 3 SNP located on the TTR gene.

The association between genotype and serum concentration for Hp indicates that there is a genetic component to the control of Hp baseline concentrations. It is possible that all 3 SNP influence Hp concentrations, however, due to the high LD measurements and that none of the SNP are within coding regions of the genes, it is more likely that they are linked to one or more SNP that do change protein expression levels. This association may prove useful because we can use genotyping information to identify animals with low and high baseline concentrations and investigate the effects of breeding for other immune or performance traits on them.

The lack of association between line and Hp is not surprising considering that no differences in serum concentration between lines were reported earlier. It also means that Hp could potentially be used as a marker of health across pig populations as line effects need not be considered. The association between genotype and Hp concentration would allow animals to be selected for either a high or a low baseline concentration and enable a comparative study of the response of the animals under disease challenge. It is likely that it is the response of an animal to infection rather than the initial baseline concentrations that will have implications for future breeding strategies.

The lack of association between genotype and APP concentration for CRP, Pig-MAP and TTR was surprising. This was particularly true in CRP where evidence from human studies made it a likely candidate for associations to be found with. However, there were multiple associations between line and APP concentration for CRP, Pig-MAP and TTR. This appears to indicate that there are intrinsic differences between the lines in APP regulation and expression. These differences are normally indicative of genetic control, but this has not been shown in this study. It should still be considered possible that associations may exist amongst the SNP not tested, particularly as the SNP tested for were all in high LD with one another and this may be an area to be explored at a later time. Alternatively, it is possible that baseline levels of CRP, Pig-MAP and TTR are associated with other genes contributing to the immune response such as the cytokine genes, IL-6 and IL-1.

This is the first study to show that Hp serum concentration in boar is associated with Hp genotype. This may prove useful in future breeding strategies or if Hp is used as a marker of herd health. In addition, it has shown that the SNP genotyped in CRP, Pig-MAP and TTR genes are not associated with serum concentrations of these proteins; however, there are associations between pig line and APP serum concentration. These results suggest that the concentrations of these proteins are under some form of genetic control.

Chapter VI

6. Concentrations and Heritability Estimates of Porcine Acute Phase Proteins in a Pig Population

6.1. Components of Variation

When studying a phenotypic trait, such as APP serum concentrations, it is often described in terms of the trait's variance. Variance can be described as the sum of the squares of the deviation between an individual measurement and the group mean. The total amount of variance (V_P) in a phenotypic trait is the sum of the variance caused by all the individual components. These include the non-genetic component (environment, V_E) and the genetic components (V_G) which is composed of the additive genetic component (V_A), the dominant genetic component (V_D) and the interaction between genes (V_I). This can be expressed as:

$$V_P = V_G + V_E$$

$$= V_A + V_D + V_I + V_E$$

By separating the components of variance, it is possible to estimate the importance of the various components, in particular, the effects of environment versus genetics. The importance of genetics in determining V_P is the heritability of the trait (Falconer and Mackay, 1996).

6.1.1. Heritability

Heritability can be described in two ways; *heritability in the broad sense* and *heritability in the narrow sense*. Heritability in the broad sense (H^2_B) is the extent to which genotype determines phenotype in a particular population in a particular environment;

$$H^2_B = V_G/V_P$$
$$= V_G/(V_E + V_G)$$

Heritability in the narrow sense or heritability (h^2) is the additive component of genetic variance and is passed on from parent to offspring;

$$h^2 = V_A/V_P$$

This is of great importance in animal breeding programmes as it determines the extent to which the offspring will resemble their parents. It is measured on a 0 - 1 scale (this can also be expressed as a percentage) with the higher value indicating higher heritability (Burton *et al.*, 2005, Falconer and Mackay, 1996).

6.1.2. Estimating Heritability

There are several methods for estimating heritability; all use the resemblance between relatives as a basis. Three of the most common methods are now described.

6.1.2.1. Regression Analyses

This is a relatively simple analysis in which a trait is measured in both offspring and parents and compared using regression analysis. The slope of regression of offspring on parents gives the h^2 estimate. Parent-offspring regression can be performed as a one-parent test where either the mother or father is compared with the offspring to give an estimate which is half of the heritability or as a mid-parent-offspring test where the two parents are averaged and which gives an estimate of the total heritability (Falconer and Mackay, 1996).

6.1.2.2. Analysis of Variance

In this method, the phenotypic variance is divided into components attributed to the differences between the progeny of different males, differences between the progeny of females mated to the same males, differences between the progeny of females mated to different males and differences between progeny of different females. An ANOVA test is then performed. These tests are often utilised in full-sib designs where siblings share sire and dam and gives an estimate which is equal to or greater than the heritability or half-sib designs where siblings share one parent and gives an estimate of a quarter of the heritability (summarised in Table 6.1) (Falconer and Mackay, 1996).

Table 6.1: Composition of phenotypic covariances and heritability estimations.

Relatives	Covariance	Regression (b) or correlation (t)
Offspring and one parent	$\frac{1}{2} V_A$	$b = \frac{1}{2} h^2$
Offspring and mid-parent	$\frac{1}{2} V_A$	$b = h^2$
Half sib	$\frac{1}{4} V_A$	$t = \frac{1}{4} h^2$
Full sib	$\frac{1}{2} V_A - \frac{1}{4} V_D + V_E$	$t = \geq \frac{1}{2} h^2$

(Falconer and Mackay, 1996)

6.1.2.3. Maximum Likelihood Analyses

As populations usually contain a number of different classes of relatives, an analysis is required that can simultaneously estimate the resemblance between individuals and calculate the relationships between them. The method of choice in animal breeding research for this analysis is termed 'restricted maximum likelihood' (REML). It is a method of estimating variance and covariance components and has the advantages of not requiring a balanced design and can cope with missing data (Patterson and Thompson, 1971). Several statistical packages are equipped to use REML including SAS 9.1 and ASREML; ASREML, in particular, is a frequently used program and was designed specifically for use in analysing breeding data (Gilmour *et al.*, 1995).

6.1.3. Experimental Design

In order to estimate heritability accurately, it is necessary to consider the design of the experiment and the method. Often the method selected is influenced by practical constraints but experimental design is an area which can be altered. In the case of heritability studies, experimental design involves the number of samples and the

relationship between them, which can be affected by cost, availability of space, labour or animals available (Falconer and Mackay, 1996). In an ideal study, the number of samples should be sufficient where an effect is of scientific significance to be also statistically significant. This not only provides statistically valid results but is also economical (prevents waste of resources) and ethical, especially where animal or human subjects are used (Lenth, 2001). There are different approaches to determining sample size, however the most popular is to determine the 'power' of a test of a hypothesis.

6.1.3.1. Statistical Power

Statistical power is the probability of obtaining a statistically significant result using a statistical test in a properly run study. It is affected by 3 parameters; the significance level (α) of the test, the sample size (n) and the actual effect size (θ) (Bausell and Li, 2002). In order to determine sample size, a power analysis is carried out involving the following;

- specifying a hypothesis test
- specifying the significance level of a test
- specifying an effect size
- obtaining historical values or an estimate of the parameters needed to complete the test
- specify a target value of the power of the test

(Lenth, 2001).

There are various procedures within statistical packages such as SAS 9.1 or on-line procedures (e.g. Java applets for power and sample size) that will carry out sample-size calculations based upon pilot studies or historical data (Lenth, 2007).

The aim of this study was to determine heritability estimates for the serum concentrations of Hp, CRP, Pig-MAP and TTR in a high-health pig population.

6.2.Methods and Materials

6.2.1. Animals

A population of 297 (120 male, 177 female) were provided for this study by Genus Plc., (PIC). The pigs were born between July and September 2005 and were bled at approximately 7 months of age. The pigs were from 10 sires bred to approximately 6 dams (pure lines) each with the number of piglets from each sire ranging from 23 to 44. The sires were from 7 pig lines representing current commercial sire and dam genetics;

- Line A: Landrace (1 sire)
- Line B: LW (1 sire)
- Line C: Duroc (2 sires)
- Line D: Synthetic pure line (75% Duroc, 25% LW) (1 sire)
- Line E: Synthetic pure line (Hampshire based) (1 sire)
- Line F: Synthetic pure line bred for growth (1 sire)
- Line G: Synthetic pure line bred for growth (derived from Line F) (3 sires)

6.2.2. Experimental Design

Pigs were born on the BLU unit which consists of breeding, gestation, farrowing and nursery units. All pigs were processed within 24 hr of birth; each pig was weighed, identified with ear tags, a tissue sample taken for DNA and given an iron dextran injection. At weaning (approx 16 days), pigs were transferred to nursery units where they were housed until 70 days of age. At this time pigs were put 'on-test' (described in Chapter 3); pigs were screened and the best pigs grouped by line and sex and transferred

to the MTG unit. Pigs were bled on one occasion on either BLU (n=238) or on MTG (n=59).

6.2.3. Farm Health Status

The farms were all considered to be of a high health status and were porcine reproductive and respiratory syndrome (PRRS) negative, *M. hyopneumoniae* negative and swine influenza virus (SIV) negative. Boars at site 1A (Figure 3.1) were routinely vaccinated for *H. parasuis*, erysipelas and ileitis and for erysipelas and ileitis at site 1B. Monthly bleeds were carried out by a licensed veterinarian in order to check for notifiable diseases and for certification purposes.

6.2.4. Blood Collection

Blood was collected by a licensed veterinarian by puncturing the external jugular vein and collecting into anticoagulant (EDTA). Tubes were mixed several times and stored on ice until returned to the laboratory. Upon return, tubes were centrifuged at 800 xg for 10 min and the plasma decanted and frozen at -80°C until shipment on ice to the UK. On arrival in the UK, tubes were immediately stored at -20°C until required for analysis.

6.2.5. Determination of Acute Phase Protein Concentration

The concentration of Hp, CRP, Pig-MAP and TTR were determined as described in Sections 3.2.5.1 – 3.2.5.4. Internal controls were ran in each assay.

6.2.6. Data Analysis

6.2.6.1. Pilot Trial for Full Trial Sample Estimation

In order to determine the number of samples required to obtain a statistically significant result, a pilot trial was carried out using the log-transformed Hp concentrations of 155 progeny from 5 sires. The data was analysed using a sire model with the PROC MIXED program of SAS 9.1 with farm as a fixed effect and sire and dam as random effects. The covariance estimates from SAS were then used to calculate heritability using the $t=h^2/4$ relationship.

To determine the number of samples required in the full scale study, a power analysis was performed using http://www.math.uiowa.edu/~rlenth/Power/ (Lenth, 2006) using data from the pilot trial.

6.2.6.2. Statistical Analysis

All data was log-transformed in order to render skewed data as close to normal distributions as possible. Data was initially analysed using a sire model with the PROC MIXED program of SAS 9.1 (SAS, 2004) in order to investigate and identify fixed effects.

6.2.6.3. Heritability Estimation

Analysis was carried out using ASReml v2 software (Gilmour *et al.*, 1995) and log transformed data. Each APP was analysed separately and variance components and genetic parameters were estimated using a restricted maximum likelihood (REML) method. An animal model was used including line, sex, farm as fixed effects with animal and dam as random effects. The heritability was then estimated using the ratio of animal variance to phenotypic variance.

6.3.Results

6.3.1. Precision of APP Tests

As the APP tests were run alongside those of Sections 3.2.5.1 – 3.2.5.4, the CV values have been included in Table 3.1.

6.3.2. Serum Concentrations of APP in a High Health Pig Herd

Serum concentrations of Hp, CRP, Pig-MAP and TTR across a high health herd are shown in Figures 6.1 – 6.4 respectively. The mean Hp, CRP and Pig-MAP concentrations were 0.65 g/L (range = 0.02 - 8.05 g/L), 214.7 mg/L (range = 8.6 - 753.6 mg/L) and 0.77 mg/ml (range = 0 – 5.52 mg/ml) respectively. The mean TTR concentration was 275.2 μg/ml (range = 41.5 – 785.4 μg/ml). Summary values are shown in Table 6.2.

Table 6.2: Summary values of Hp, CRP, Pig-MAP and TTR concentrations in a high health pig herd.

	Hp (g/L)	CRP (mg/L)	Pig-MAP (mg/ml)	TTR (μg/ml)
n	297	297	297	297
Min.	0.02	8.6	0.00	41.5
Max.	8.05	753.6	5.52	785.4
Median	0.50	202.8	0.57	258.3
Mean	0.65	214.9	0.77	275.2
SEM	0.04	7.11	0.04	9.28
SD	0.72	122.56	0.71	159.99

Abbreviations: n: no. of pigs. SEM: standard error of the mean. SD: standard deviation.

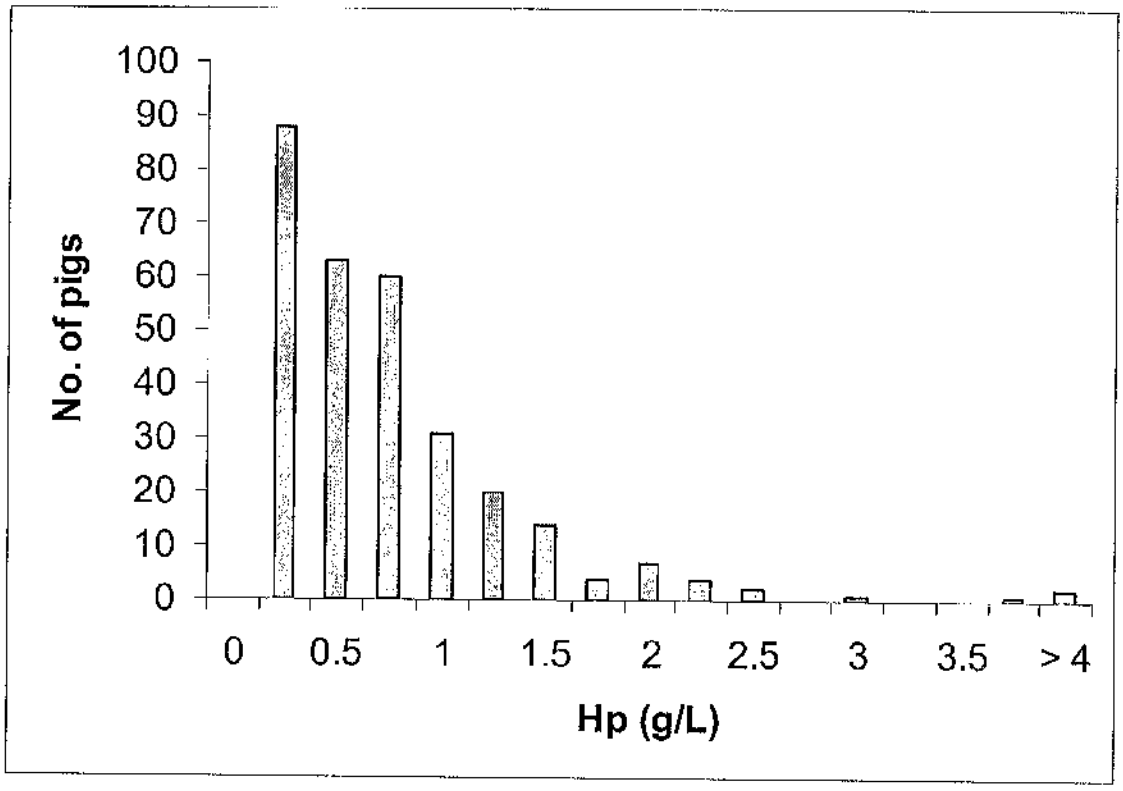


Figure 6.1: Serum concentrations of Hp (g/L) across a high health pig herd (n = 297).

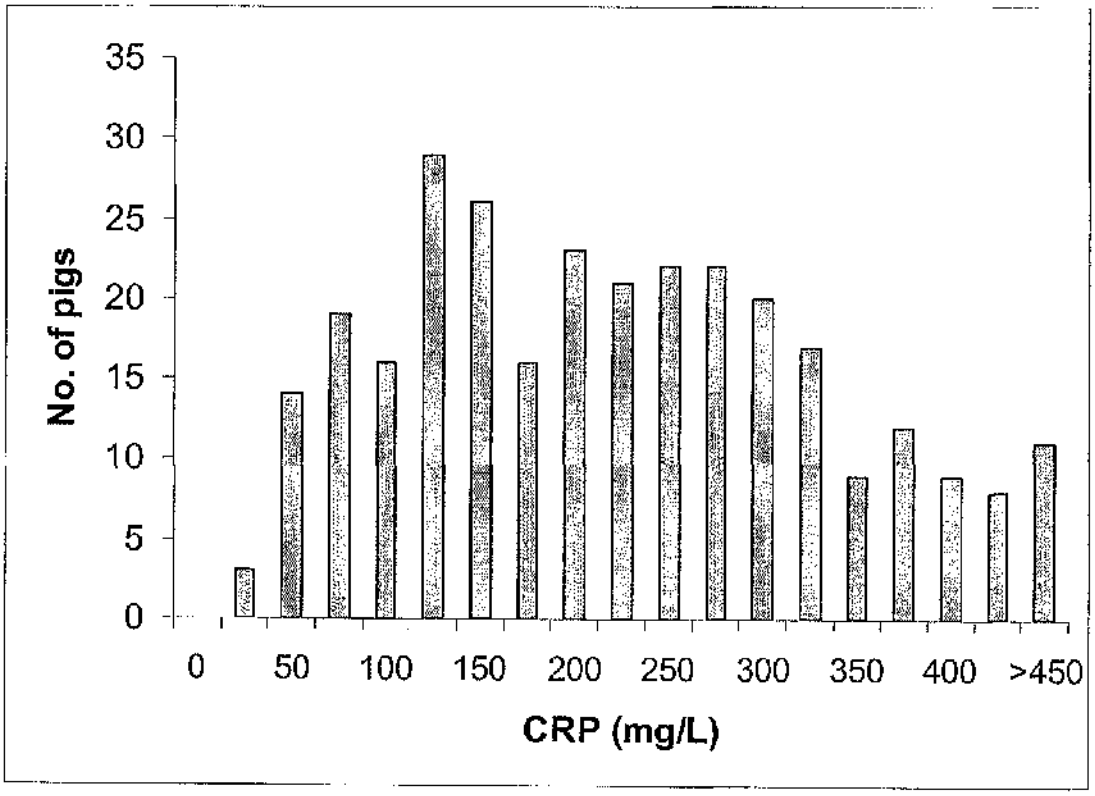


Figure 6.2: Serum concentrations of CRP (mg/L) across a high health pig herd (*n* = 297).

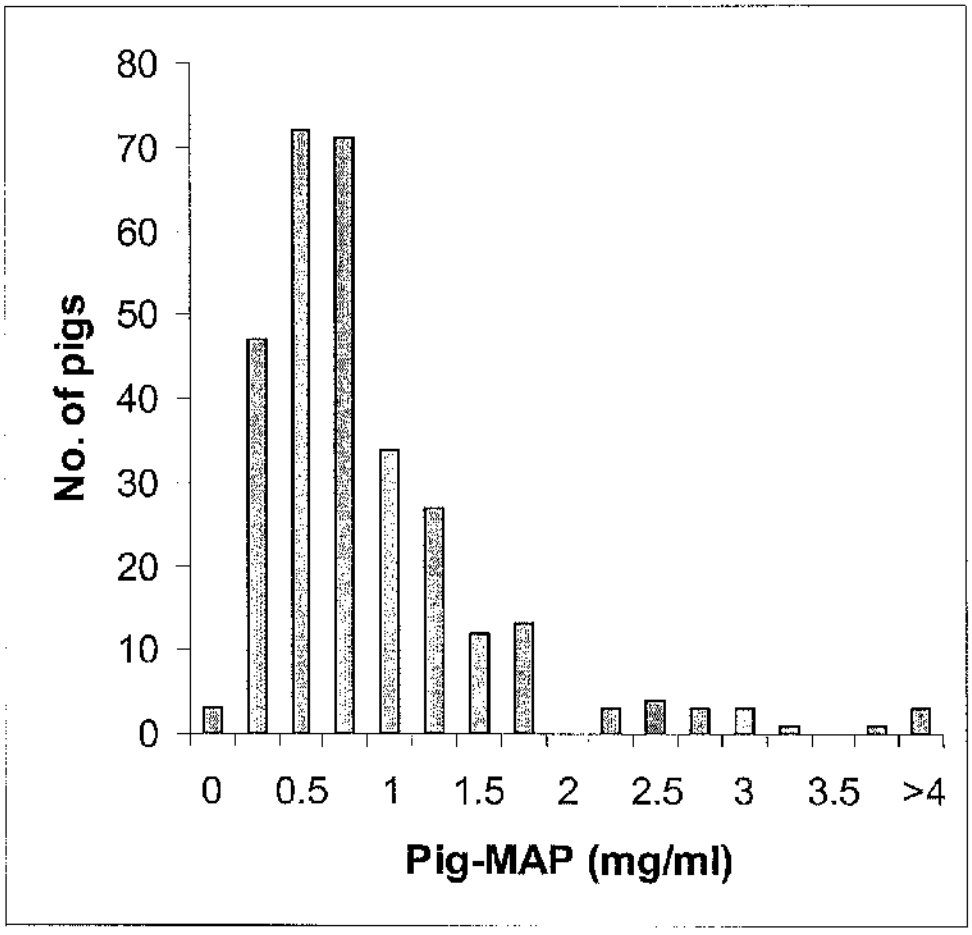


Figure 6.3: Serum concentrations of Pig-MAP (mg/ml) in a high health pig herd (*n* = 297).

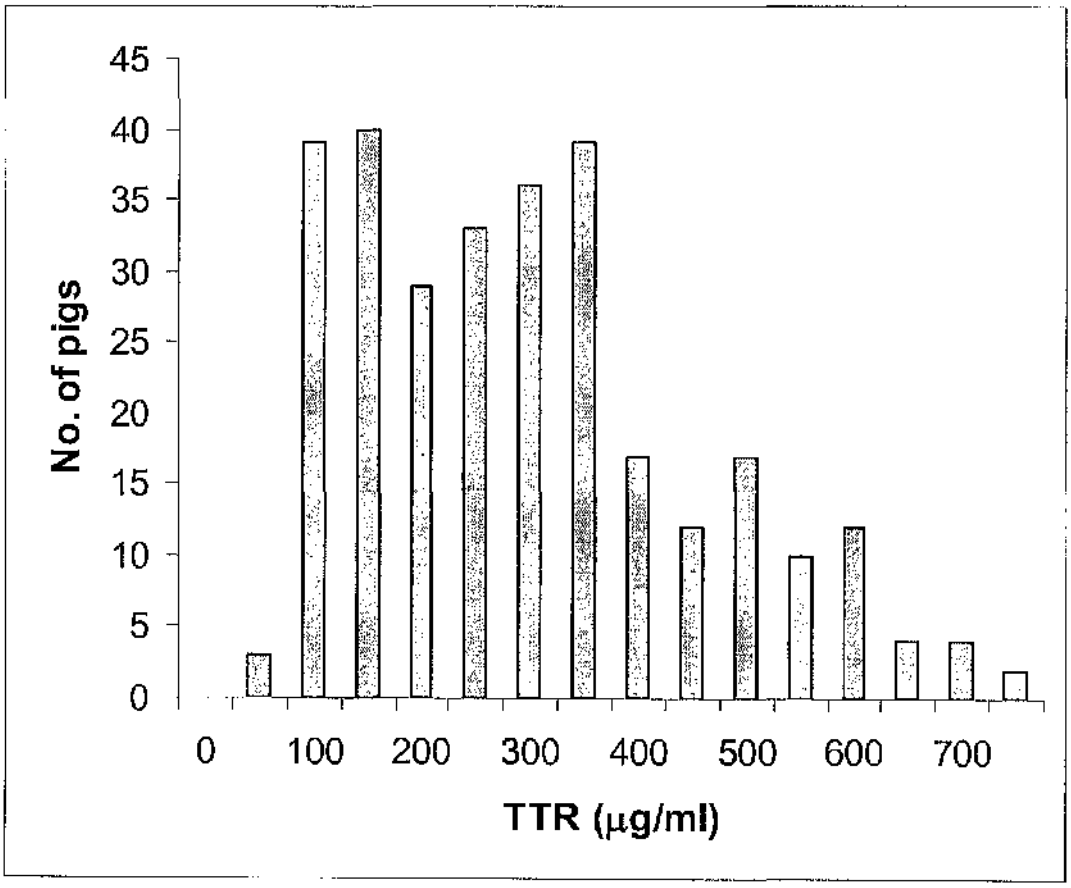


Figure 6.4: Serum concentrations of TTR (µg/ml) in a high health pig herd (n = 297).

6.3.3. Comparison of Serum Concentrations of APP between Sexes

Serum concentrations of Hp, CRP, Pig-MAP and TTR for male and females are shown in Figures 6.5 – 6.8. Summary values are given in Table 6.3. There was a significant difference ($p < 0.05$) in serum Hp concentrations between male and female pigs with female pigs exhibiting higher concentrations of Hp. The difference in serum concentrations of CRP in male and female pigs, with the latter having a higher concentration, was approaching significance at $p=0.06$. No significant difference was found between males and females for Pig-MAP and TTR.

6.3.4. Comparison of Serum Concentrations between Farm Units

Serum concentrations of Hp, CRP, Pig-MAP and TTR for both units (BLU and MTG) are shown in Figures 6.9 – 6.13. Summary values are given in Table 6.4. There was a significant difference ($p < 0.05$) in serum CRP concentrations between the two units with higher CRP concentrations found on the BLU unit. No significant differences were found between units for Hp, Pig-MAP and TTR.

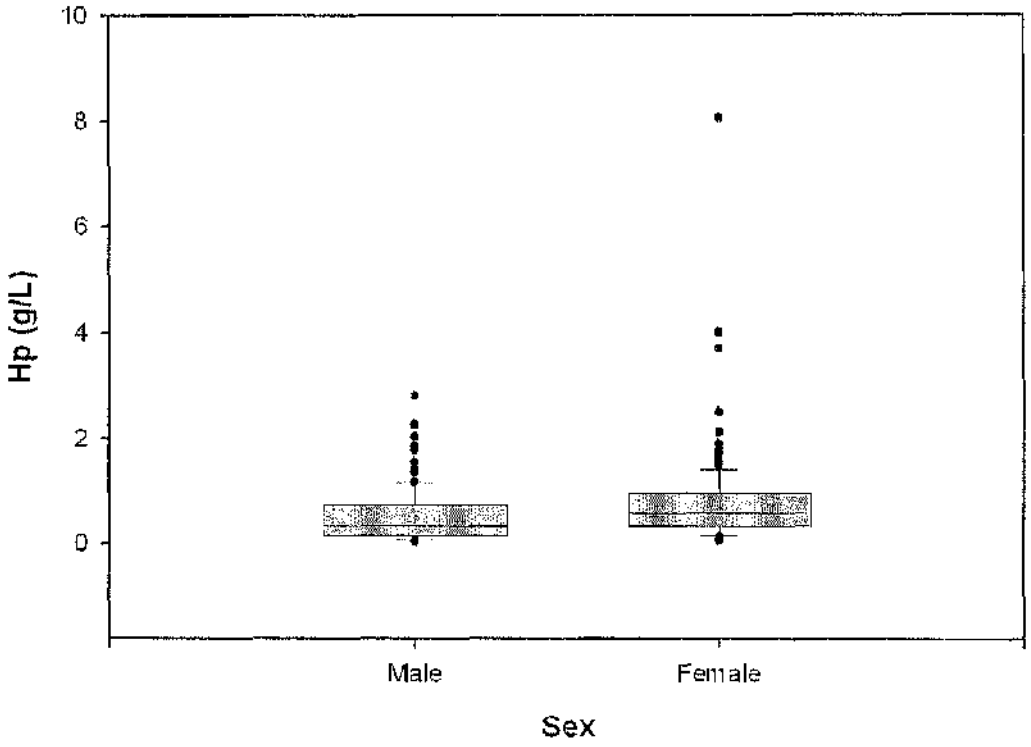


Figure 6.5: Hp concentrations (g/L) in male (n=120) and female pigs (n=177). The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

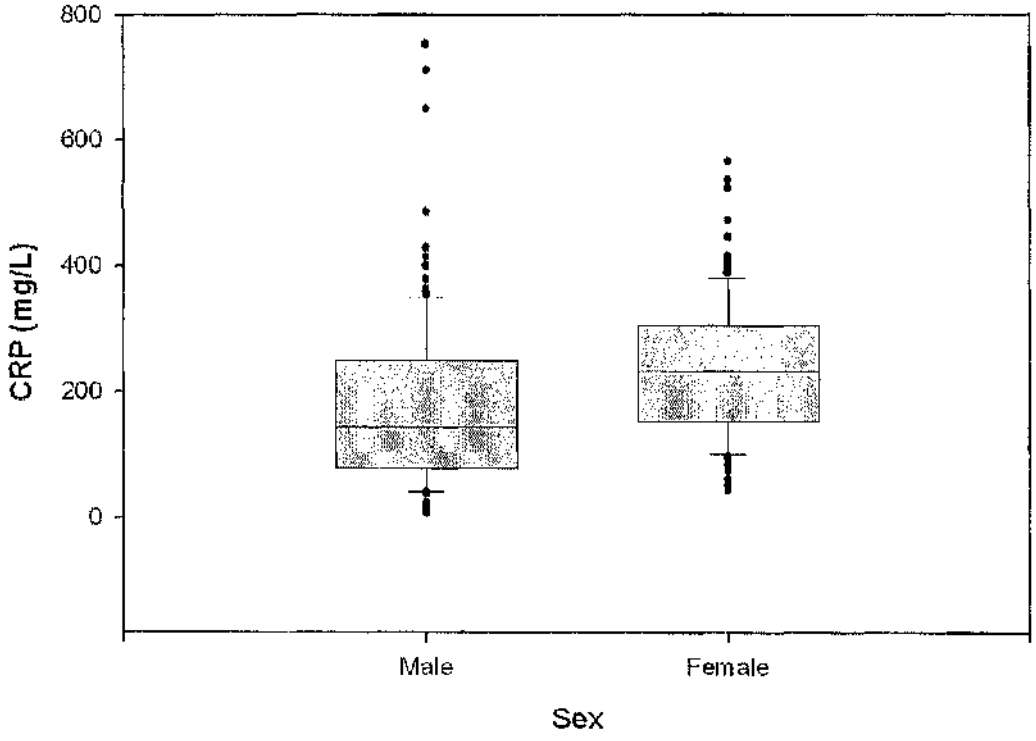


Figure 6.6: CRP concentrations (mg/L) in male (n=120) and female pigs (n=177). The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

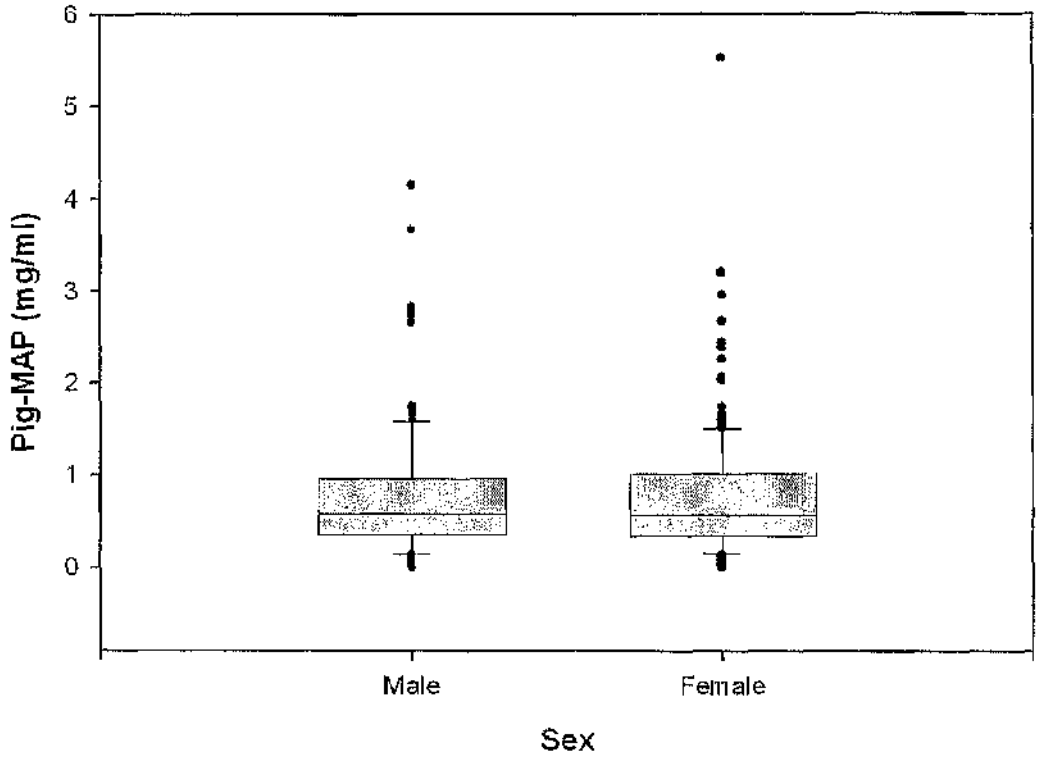


Figure 6.7: Pig-MAP concentrations (mg/ml) in male (n=120) and female pigs (n=177). The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

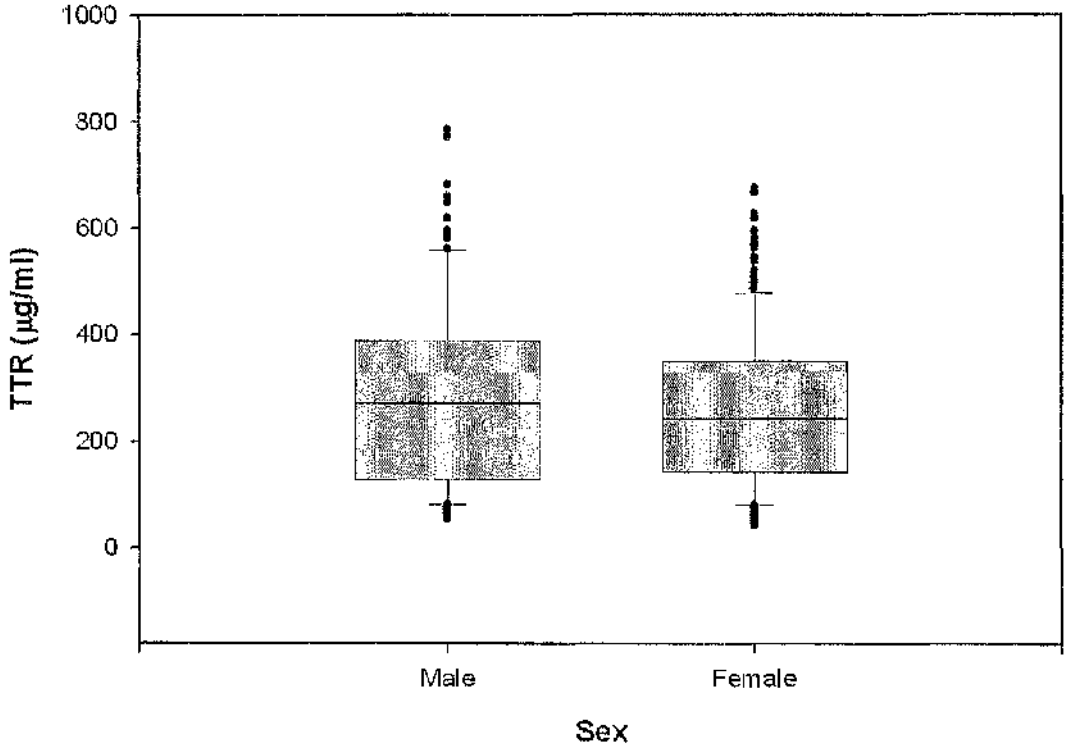


Figure 6.8: TTR concentrations (µg/ml) in male (n=120) and female pigs (n=177). The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers.

Table 6.3: Summary values of Hp, CRP, Pig-MAP and TTR concentrations between male and female pigs in a high health herd.

	Hp (g/L)		CRP (mg/L)		Pig-MAP mg/ml)		TTR μg/ml)	
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**
n	120	177	120	177	120	177	120	177
Min.	0.02	0.03	8.6	43.1	0.00	0.00	52.9	41.5
Max.	2.80	8.05	753.6	566.3	4.15	5.52	785.4	675.7
Median	0.32	0.58	145.0	234.7	0.58	0.57	270.3	242.5
Mean	0.50	0.76	182.3	237.0	0.79	0.76	288.8	266.1
SEM	0.05	0.06	12.42	8.07	0.07	0.05	16.24	11.01
SD	0.52	0.81	136.11	107.34	0.76	0.68	177.92	146.42

Abbreviations: M: Male. F: Female. N: no. of pigs. SEM: standard error of the mean. SD: Standard deviation.

Table 6.4: Summary values of Hp, CRP, Pig-MAP and TTR concentrations across 2 high health farm units.

	Hp (g/L)		CRP (mg/L)		Pig-MAP mg/ml)		TTR μg/ml)	
	BLU	**MTG**	**BLU**	**MTG**	**BLU**	**MTG**	**BLU**	**MTG**
n	238	59	238	59	238	59	238	59
Min.	0.02	0.02	40.3	8.6	0	0.01	41.5	52.9
Max.	8.05	2.28	712.1	753.6	5.52	3.66	675.7	785.4
Median	0.53	0.33	220.8	137.0	0.61	0.52	228.1	324.0
Mean	0.69	0.50	229.0	157.8	0.80	0.63	250.4	375.6
SEM	0.05	0.06	7.61	16.56	0.05	0.08	9.55	22.21
SD	0.76	0.45	117.42	127.22	0.73	0.65	147.38	170.60

Abbreviations: BLU: Bluegrass. MTG: Mt. Gilead. N: no. of pigs. SEM: standard error of the mean. SD: Standard deviation.

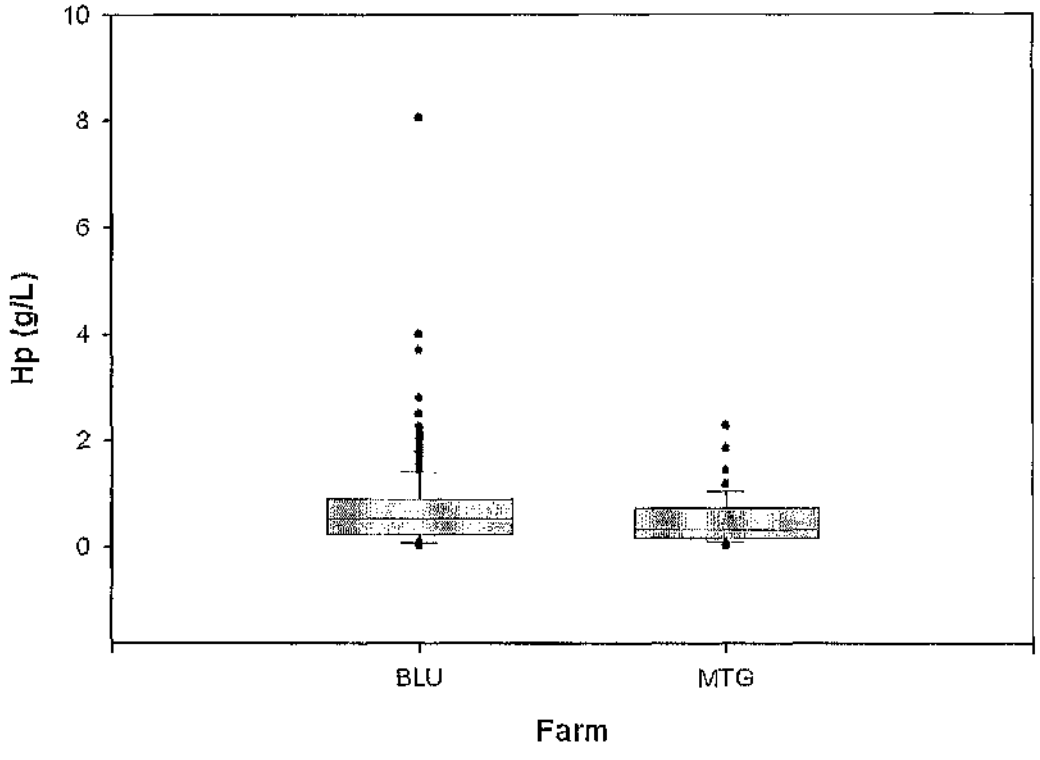


Figure 6.9: Hp concentration (g/L) across two high health pig units. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. BLU: Bluegrass (n= 238). MTG: Mt. Gilead (n=59).

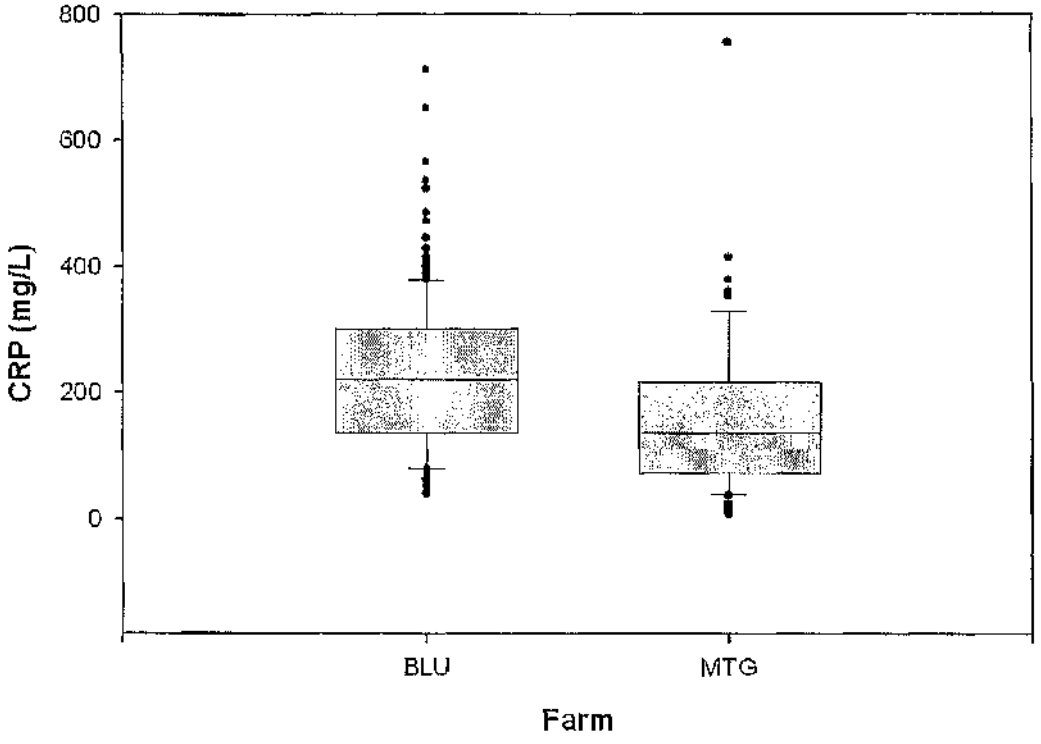


Figure 6.10: CRP concentrations (mg/L) across two high health pig units. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. BLU: Bluegrass (n= 238). MTG: Mt. Gilead (n=59).

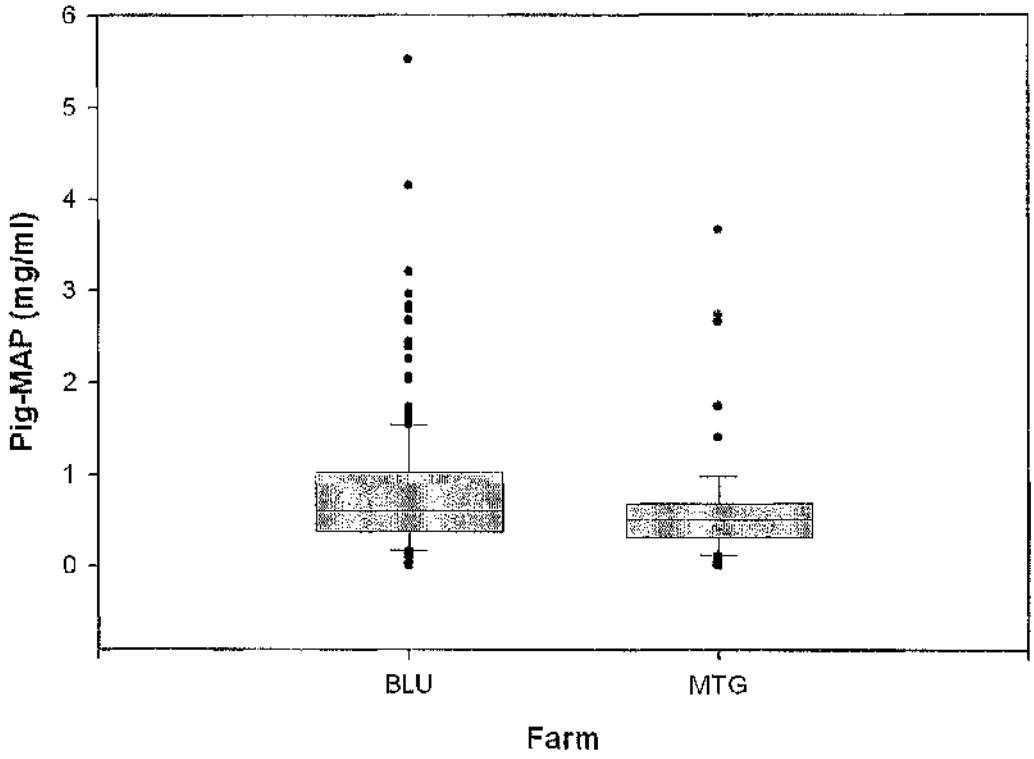


Figure 6.11: Pig-MAP concentrations (mg/ml) across two high health pig units. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. BLU: Bluegrass (n= 238). MTG: Mt. Gilead (n=59).

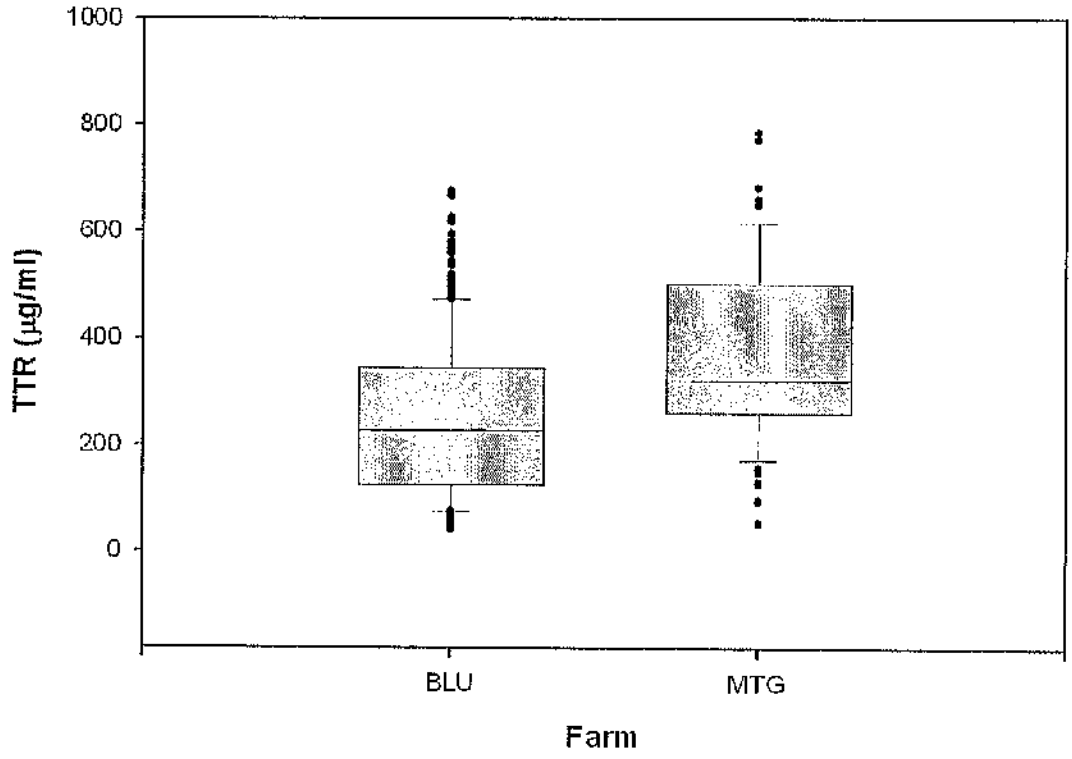


Figure 6.12: TTR concentrations (µg/ml) across two high health pig units. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. BLU: Bluegrass (n= 238). MTG: Mt. Gilead (n=59).

6.3.5. Comparison of Serum Concentrations of APP across Pig Lines

Serum concentrations of Hp, CRP, Pig-MAP and TTR in all lines are shown in Figures 6.13 - 6.16 respectively. Summary values are given in Tables 6.5 – 6.8. There was a significant difference found between lines for Hp ($p < 0.0001$), CRP ($p = 0.0001$) and TTR ($p < 0.0001$). There was no significant difference in Pig-MAP concentration between pig lines. Individual significance values for the lines can be found in Tables 6.9 – 6.11.

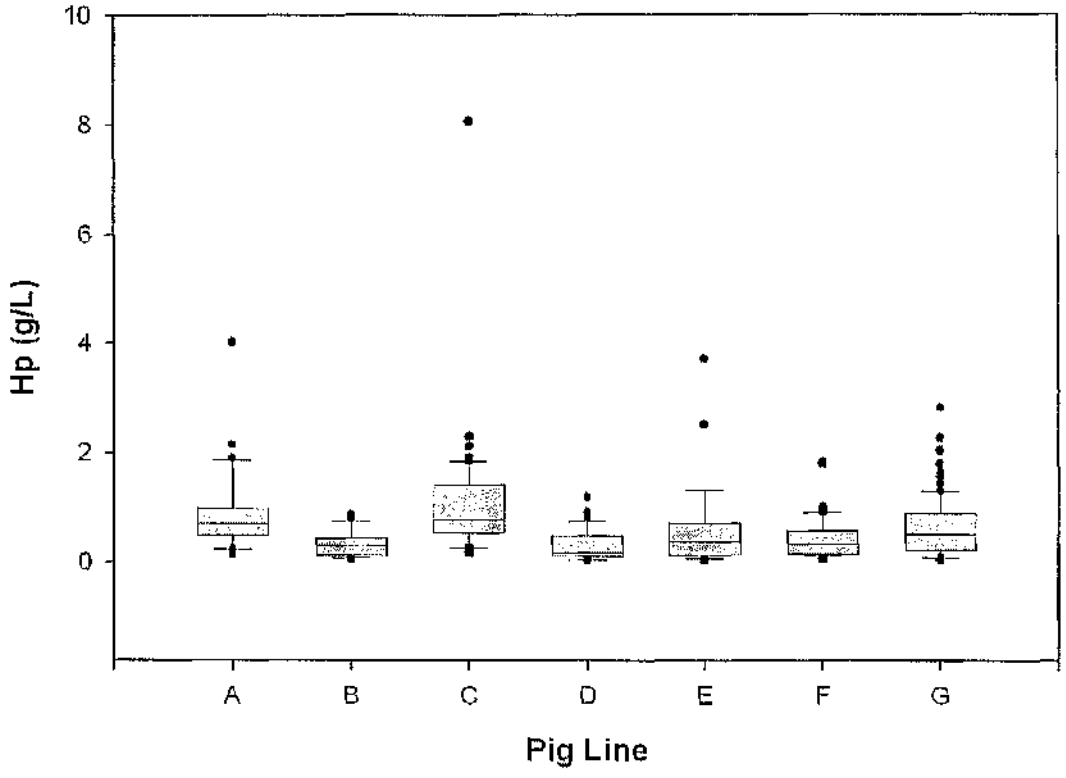


Figure 6.13: Hp concentrations (g/L) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

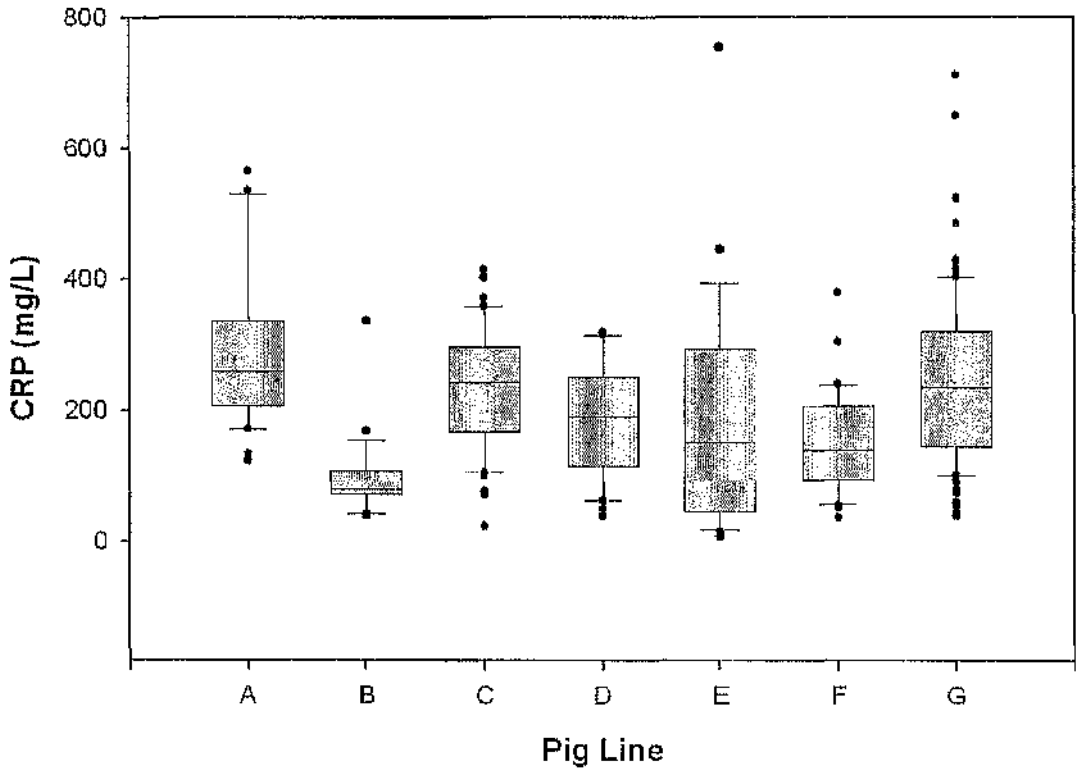


Figure 6.14: CRP concentrations (mg/L) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

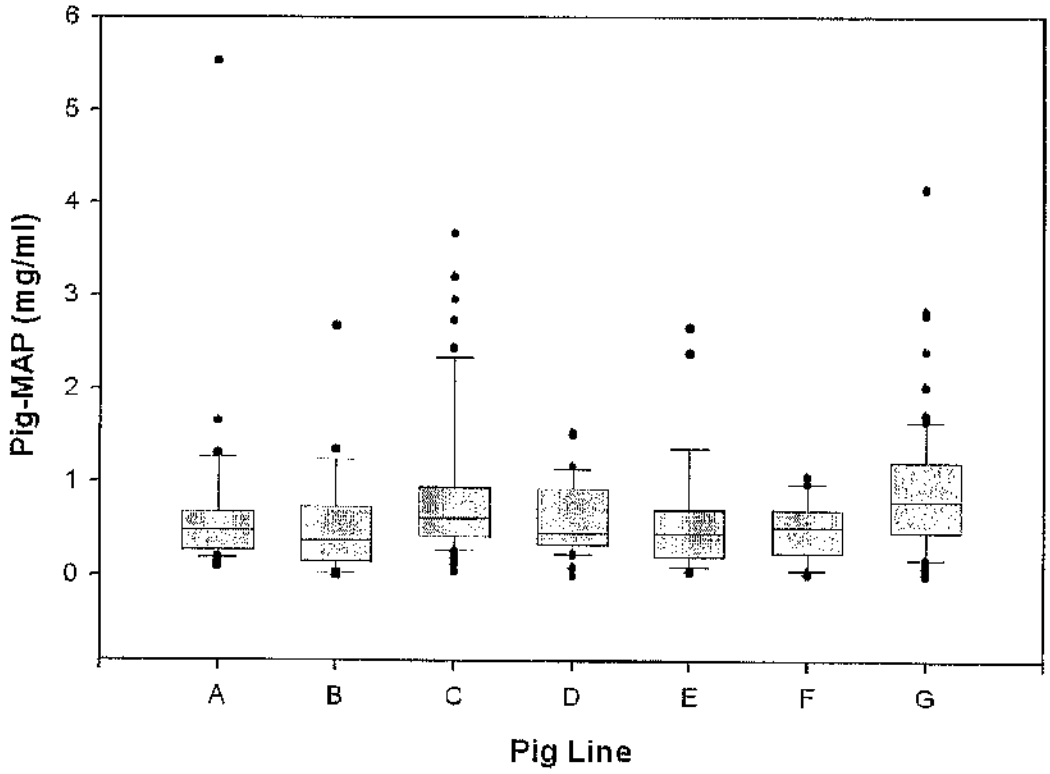


Figure 6.15: Pig-MAP concentrations (mg/ml) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

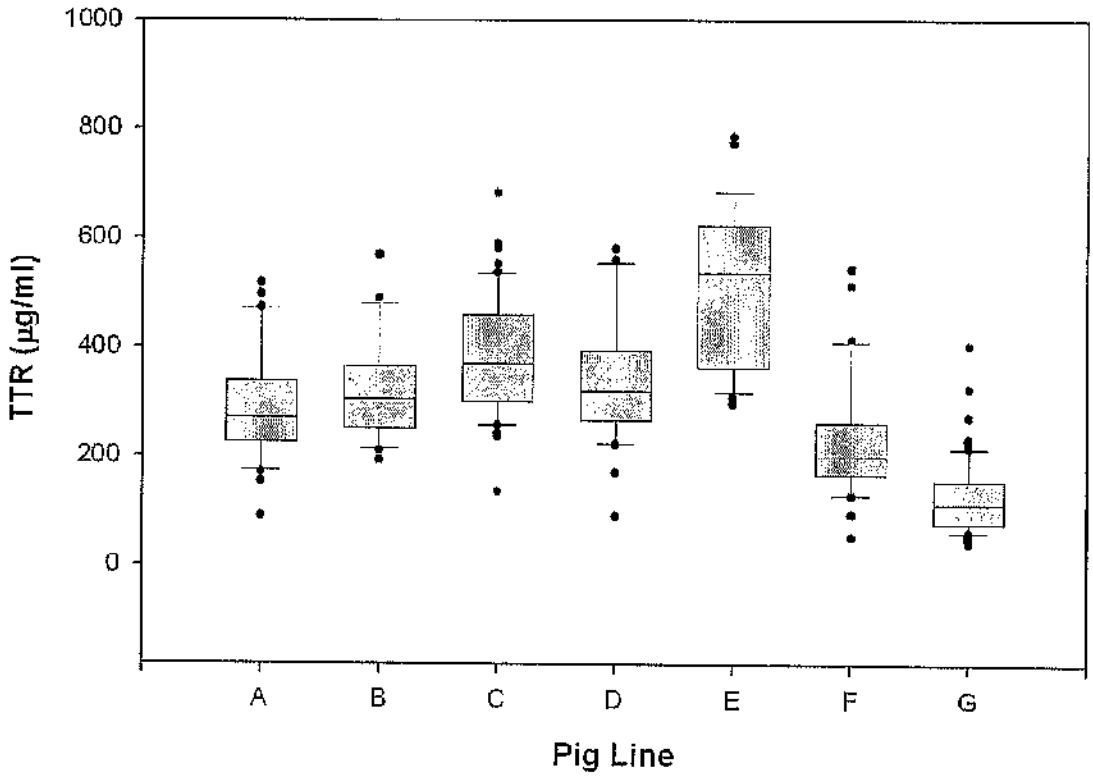


Figure 6.16: TTR concentrations (µg/ml) across 7 pig lines. The line within the box marks the median; the boundaries of the box represent the 25th and 75th percentiles; whiskers above and below the box indicate the 10th and 90th percentile and the points outside the end of the whiskers are outliers. Line A: Landrace; Line B: LW; Line C: Duroc; Line D: Synthetic pure line; Line E: Synthetic pure line; Line F: Synthetic pure line bred for growth; Line G: Synthetic pure line bred for growth.

Table 6.5: Summary values of Hp concentrations across 7 pig lines (n=297).

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	30	23	54	32	28	30	100
Min.	0.13	0.03	0.16	0.03	0.02	0.04	0.02
Max	4.00	0.85	8.05	1.19	3.70	1.80	2.80
Median	0.71	0.31	0.78	0.18	0.38	0.34	0.63
Mean	0.88	0.33	1.04	0.30	0.62	0.42	0.51
SEM	0.14	0.05	0.15	0.05	0.15	0.07	0.05
SD	0.75	0.23	1.10	0.29	0.80	0.36	0.53

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation

Table 6.6: Summary values of CRP concentrations across 7 pig lines (n=297).

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	30	23	54	32	28	30	100
Min.	124.2	39.2	25.1	40.3	8.6	37.3	40.8
Max	566.3	336.6	414.9	318.6	753.6	379.9	712.1
Median	259.9	81.0	242.3	190.9	151.3	140.7	235.8
Mean	291.5	97.5	234.8	181.8	187.5	149.5	246.0
SEM	21.81	12.72	12.42	15.23	31.70	14.39	12.49
SD	119.43	61.01	91.25	86.14	167.75	78.83	124.88

Abbreviations: *n*: no. of pigs; SEM: standard error of the mean. SD: standard deviation

Table 6.7: Summary values of Pig-MAP concentrations across 7 pig lines (n=297).

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	30	23	54	32	28	30	100
Min.	0.09	0.01	0.05	0.00	0.05	0.02	0.00
Max	5.52	2.68	3.67	1.54	2.67	1.06	4.15
Median	0.49	0.39	0.63	0.46	0.46	0.53	0.80
Mean	0.71	0.57	0.90	0.63	0.60	0.51	0.93
SEM	0.18	0.12	0.11	0.07	0.12	0.06	0.07
SD	0.97	0.58	0.82	0.38	0.63	0.31	0.74

Abbreviations: n: no. of pigs; SEM: standard error of the mean. SD: standard deviation.

Table 6.8: Summary values of TTR concentrations across 7 pig lines (n=297).

	Line A	Line B	Line C	Line D	Line E	Line F	Line G
n	30	23	54	32	28	30	100
Min.	90.3	192.0	136.0	89.6	296.5	52.9	41.5
Max	517.8	568.9	682.1	579.7	785.4	544.3	403.4
Median	271.1	306.1	370.2	321.4	536.9	201.2	114.1
Mean	287.5	323.5	381.7	341.4	519.8	229.9	127.0
SEM	18.18	19.38	14.52	21.00	27.28	20.09	6.36
SD	99.58	92.93	106.69	118.82	144.33	110.05	63.57

Abbreviations: n: no. of pigs; SEM: standard error of the mean. SD: standard deviation

Table 6.9: Significance values for the difference in Hp concentrations across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line H
Line A	.	0.0014**	0.0872	<0.0001***	0.1774	0.0239*	0.0349*
Line B	0.0014**	.	<0.0001***	0.4925	0.0630	0.3121	0.0500*
Line C	0.0872	<0.0001	.	<0.0001***	0.0007***	<0.0001***	<0.0001***
Line D	<0.0001***	0.4925	<0.0001***	.	0.0097**	0.0834	0.0015**
Line E	0.1774	0.0630	0.0007***	0.0097**	.	0.3405	0.7670
Line F	0.0239*	0.3121	<0.0001***	0.0834	0.3405	.	0.4323
Line H	0.0349*	0.0500*	<0.0001***	0.0015**	0.7670	0.4323	.

*p < 0.05, **p < 0.01, ***p < 0.001

Table 6.10: Significance values for the differences in CRP concentrations across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line H
Line A	.	<0.0001***	0.7412	0.0469*	0.0225*	0.0477*	0.3298
Line B	<0.0001***	.	<0.0001***	0.0247*	0.0522	0.0270*	<0.0001***
Line C	0.7412	<0.0001***	.	0.0621	0.0194*	0.0469*	0.4707
Line D	0.0469*	0.0247*	0.0621	.	0.7404	0.9707	0.1370
Line E	0.0225*	0.0522	0.0194*	0.7404	.	0.7603	0.0647
Line F	0.0477*	0.0270*	0.0469*	0.9707	0.7603	.	0.1397
Line H	0.3298	<0.0001***	0.4707	0.1370	0.0647	0.1397	.

*p < 0.05, **p < 0.01, ***p < 0.001

Table 6.11: Significance values for the differences in TTR concentrations across 7 pig lines.

	Line A	Line B	Line C	Line D	Line E	Line F	Line H
Line A	.	0.6265	0.0819	0.3900	0.0043**	0.2423	<0.0001***
Line B	0.6265	.	0.2751	0.7504	0.0261*	0.1078	<0.0001***
Line C	0.0819	0.2751	.	0.4266	0.1257	0.0015**	<0.0001***
Line D	0.3900	0.7504	0.4266	.	0.0429*	0.0413*	<0.0001***
Line E	0.0043**	0.0261*	0.1257	0.0429*	.	<0.0001***	<0.0001***
Line F	0.2423	0.1078	0.0015	0.0413*	<0.0001***	.	0.0007***
Line H	<0.0001***	<0.0001***	<0.0001	<0.0001***	<0.0001***	0.0007***	.

*p < 0.05, **p < 0.01, ***p < 0.001

6.3.6. Sample Size Estimation

Using the covariance data from SAS 9.1, heritability was initially estimated at 1 (100%). However, farm and farm*sire effects were not included at this point.

The power analysis showed that with the use of 10 sires, 4 dams and 20 offspring per sire there was an 84% chance of detecting a significant sire variation. These numbers were used in order to obtain enough samples for the full scale trial.

6.3.7. Heritability

The heritability was estimated using the ratio of animal variance to phenotypic variance, this gave heritability estimates for Hp, CRP, Pig-MAP and TTR of 0.15 (0.00), 0.13 (0.04), 0.12 (0.03) and 0.07 (0.02) respectively (standard error is indicated in brackets). The estimates for Hp, CRP and Pig-MAP are considered indicative of low heritable traits. The heritability estimate for TTR is extremely low.

6.4.Discussion

The aim of this study was to estimate heritability of Hp, CRP, Pig-MAP and TTR in a high health pig population. Although the heritability of Hp had been determined previously (Henryon *et al.*, 2006), prior to this study, no heritability studies of CRP, Pig-MAP and TTR have been carried out in livestock, however similar studies have been carried out in human populations.

The pigs used in this study were a mixture of male and female and were bled on two different units. As previously, APP concentrations showed skewed distribution towards the lower ranges. However, Hp, CRP and TTR all showed higher mean concentrations than the previous studies (Chapters 3 and 5) while Pig-MAP showed a lower mean concentration. The difference in the mean concentrations could be attributed to the inclusion of female pigs in this study or the effect of the farm where the pigs were bled. Despite the differences, Hp, Pig-MAP and TTR concentrations were all within the accepted 'normal healthy' ranges (Campbell *et al.*, 2005, Martin *et al.*, 2005, Petersen *et al.*, 2004). The mean CRP concentration was approximately double that of the reported healthy range (Parra *et al.*, 2006).

Comparison of the APP concentrations between male and female pigs revealed significant differences in Hp concentrations with females exhibiting higher Hp concentrations than males. This corroborates a recent study carried out by Pineiro *et al.*, who also reported that there was a significant difference in Pig-MAP concentrations between males and females which was not seen in this study (Pineiro *et al.*, 2007a). The difference in concentrations of

CRP between male and female pigs was approaching significance with females showing higher CRP concentrations; a significant difference has been reported previously with females showing higher concentrations (Pineiro *et al.*, 2007c). The difference in baseline APP concentrations between sexes suggests that different physiological mechanisms could be involved with APP production possibly through hormonal interactions in the HPA.

Differences in CRP concentrations were also observed between the two farm units in which the pigs were bled. This may have been due to some form of stimulus occurring on the BLU unit such as changes in routine, different husbandry techniques (Pineiro *et al.*, 2007b) or because only male pigs were housed at MTG whilst male and female were housed at BLU (as discussed, female pigs in this study had higher concentrations of CRP). It is unlikely to be due to sub-clinical disease as both farms are high health units and no difference was observed in Hp, Pig-MAP or TTR.

As previously discussed in Chapter 3 and 5, differences were found between the pig lines and APP concentrations. Surprisingly, Hp showed a line difference in this study with Line C (Duroc) showing a significantly higher Hp concentration than all other lines except Line A (Landrace) whereas Line D (Synthetic line) showed a significantly lower Hp concentration than Lines A, C, E (Synthetic) and G (Synthetic). Pig-MAP did not show any line differences in contrast to the earlier studies. The differences in the studies could be attributed to the inclusion of females in this study, particularly as there are sex differences in Hp concentration. This indicates that the use of APP as biomarkers must take into account the sex of the pig herd in question; whether it is a mixed sex herd or

single sex. As so many factors appear to control herd baseline APP concentrations their use as biomarkers of health on a herd level might be more problematic than originally thought.

The pilot study of the heritability of Hp concentrations showed an extremely high heritability of 1, at this point the estimate was seen as inaccurate and was probably the result of not including farm and farm*sire factors. However, the lowering of the estimate to a more realistic 0.6 and input of the covariance data into a power and sample size analysis provided the information needed to estimate the number of samples required for the full scale heritability study. The use of this number of samples (taken to be the minimum required), meant that the full scale study was of an adequate size to identify significant effects (in this case heritability) (Lenth, 2001).

The heritability estimates were performed using ASReml with data obtained from SAS 9.1. This is common practice in order to identify fixed effects, (for example sex, line and farm) and to gain more information on the data such as least square means. Each APP was analysed using the same program in order that direct comparisons could be made of the data and this also took into account any fixed effects that could have an influence on the heritability estimate.

The heritability estimates were low for each of the APP measured which was not unexpected as heritability estimates for immune traits and/or disease resistance are generally low (Wilkie and Mallard, 2000). A previous study had found that the heritability of Hp in boars was 0.14 which is similar to the finding in this study (Henryon *et al.*, 2006),

however the heritability of CRP, Pig-MAP and TTR concentrations have never been estimated before in pigs. The low heritability estimates mean that breeding for baseline APP concentrations (whether high or low) will prove to be inefficient at the present time without good genetic markers and that alternate breeding strategies would be required. Overall the results indicate that the majority of variance in APP concentrations is due to components such as line and sex rather than the influence of SNP in the genes. This indicates that more studies are required to fully understand the sources of variation and that care must be taken when carrying out genetic studies involving the APP.

Chapter VII

7. General Discussion

The aim of this thesis was to investigate the porcine APP in a high health pig population and the relevant conclusions have been discussed in the previous chapters. This chapter will review the most significant findings and highlight areas which are worthy of future investigations.

Acute phase proteins are produced by the hepatocytes in response to infection, trauma or inflammation. They are currently used as an indicator of disease (including sub-clinical disease) and their measurement has been suggested as a biomarker of health and welfare in livestock and companion animals (Petersen *et al.*, 2004, Gruys *et al.*, 1994). The measurement of porcine APP as an indicator of underlying problems (for example, sub-clinical disease) in production systems was first suggested by Eckersall (1987), and since then a great deal of research has been performed including a major EU Shared Cost project. It has also been proposed that APP may be useful in breeding for disease resistance.

Despite the high number of studies involving porcine APP, very little was known about their control or their genetics. Previous to this study, the porcine APP genes had been mapped in pigs (Chomdej *et al.*, 2004, Ponsuksili *et al.*, 2002, Baskin *et al.*, 1998, Archibald *et al.*, 1996); however it was not known what, if any, genetic associations there may be with the APP serum concentrations as had been demonstrated in humans. This thesis set out to investigate the porcine APP in a high health setting (so as to obtain baseline

concentrations with low disease presence) and determine whether there were genetic associations.

It was first necessary to develop and validate an immunoassay for the measurement of CRP; as commercial assays were costly and development of a method for assay automation would be a boon to analytical operation. Unfortunately the immunoturbidimetric assay most suitable for automation had a limit of sensitivity too high for use with non-stimulated CRP concentrations as found in the serum from the healthy animals in this study. Although measurement of APP has developed considerably over the years, the basis of the ELISA developed and validated in this study was a relatively simple one using the CRP ligand; phosphocholine, and the cross-reactivity of anti-human CRP to pCRP. Although not ideal, this approach had considerably greater success than the use of anti-serum to pCRP in both the sandwich ELISA and immunoturbidimetric assay due to non-specificity of the antibody. Investigation of this showed that polyclonal anti-serum to pCRP produced in sheep or chicken reacted with several protein bands some of which were assumed to be aggregates of the monomer. This assumption was further strengthened by the appearance of the bands in purified pCRP following various purification protocols. Studies of human CRP have found that CRP is glycosylated and that glycosylation varies with different disease states (Das *et al.*, 2003). It is possible that this is occurring in pigs and this may account for the extra protein bands viewed in purified pCRP and in the antibody cross-reactions. Glycosylation is apparent in other APP such as AGP and can have effects on protein function which may be of importance (Hochepied *et al.*, 2003). However the extra bands could be due to non-specific cross reactions which lead to the poor correlation results

between both the sandwich ELISA and immunoturbidimetric assays and the commercial assay used for validation purposes.

In order to overcome these issues, there are two pathways that could be taken. The first would be the production of monoclonal antibodies against pCRP and use of them in an ELISA. The second would be to adapt the immunoturbidimetric assay protocol developed in Chapter 2 for use with latex beads to which a specific polyclonal or mix of monoclonal antibodies are conjugated. This would give the high specificity and sensitivity required in a robust and automated format.

The developed pCRP assay was used in Chapter 3 in an investigation of baseline concentrations of porcine APP in a boar population. 'Normal' ranges of the porcine APP have been previously published (Parra *et al.*, 2006, Campbell *et al.*, 2005, Martin *et al.*, 2005) and it was expected that the results of this study would corroborate the proposed ranges. However, although the herd mean concentrations of Hp, CRP and TTR all fell within the previously reported healthy ranges; Pig-MAP showed a mean concentration similar to one previous study (Henryon *et al.*, 2006) but almost twice that of studies earlier than 2006. This could be attributed to differing ages of the pigs in the studies, sex or breed but it demonstrates the need for APP results to be interpreted with caution, in conjunction with one another and with greater collaboration between laboratories in developing quality assurance schemes. As CRP, Pig-MAP and Hp all show significant correlations with one another, it would be possible to measure 2 of the positive APP rather than all 3. This would prove to be more cost and time effective if the most suitable APP are chosen for

measurement. Line differences were also apparent in CRP, Pig-MAP and TTR indicating that there is genetic variation in APP baseline concentrations. It also gives rise to the possibility of selecting lines to look for associations with selection traits.

Chapter 4 investigated the 4 porcine APP genes and identified 71 SNP, many of which were novel, and 17 of which were genotyped across a boar population. The results showed allele frequency differences between lines giving strength to the argument that selective breeding has had an effect on the APP. The SNP within each gene were found to be in high LD with each other, meaning that if further investigations take place it will only be necessary to genotype one or two of them per gene. The use of genotyping in animal breeding has become widespread and is an efficient way of selecting desirable animals, particularly if the phenotypic trait takes time to develop or is difficult to measure. This avoids the cost of feeding and housing animals unnecessarily and improves accuracy of selection. This is only possible if a phenotypic trait can be linked to a genotype, however SNP in LD with the causative polymorphism can be used satisfactorily (Dekkers, 2004).

Associations between Hp baseline concentrations and SNP genotype were identified between 3 of the SNP in the Hp gene. No associations were found between baseline concentrations and CRP, Pig-MAP and TTR SNP. Interestingly, there were no associations between pig line and Hp concentrations. This would make Hp better suited to being a marker of herd health in boars as no line effects need to be taken into account. The advantages of using Hp as a marker is that there is a greater understanding of the relationships between serum concentrations of Hp and disease states than with any other

APP. Additionally, the assay for Hp is robust, has been extensively validated and is efficient in coping with large number of samples; however as with all APP tests at the present time, it requires the facilities of a laboratory for analysis. If APP are to be used as markers of animal health, more research is required in order to make testing of samples more efficient and either portable or incorporated into on-farm testing procedures. This could be accomplished by the use of body fluids other than blood (for example, saliva or urine) thus removing the need for veterinary or licensed personnel, and by adapting assays into point-of-care-tests, for example, dipstick testing. The alternative is the multiplexing of assays allowing the simultaneous measurement of several APP; this would have the advantage of producing an acute phase index.

The use of CRP, Pig-MAP and TTR as biomarkers should not be discounted at the present time. Although no associations with genotype were identified in this study, the data and samples are available for further genotyping of the remaining SNP and it is possible that associations may be discovered. Pig line does has a significant association with baseline concentrations of these proteins which makes use of them as biomarkers of health more problematic as this would have to be taken into account. This does raise the question of whether APP concentrations and/or their SNP genotype are associated with production traits, as each line will have been selected for a particular trait. If this were to be the case, then measurement of the APP could provide alternative markers for production traits.

Although not tested for in this thesis, association of APP with production traits has been suggested. This is in part due to the amino acid requirements of an acute phase reaction in

which muscle proteins are catabolised as a source of energy and for the supply of amino acids (Reeds and Jahoor, 2001). Studies have also shown that immune traits and production traits are inversely correlated (Clapperton *et al.*, 2005a). If APP are found to be associated with production traits then it is possible that either high or low baseline concentrations will be the aim of selection. This thesis provides evidence that this is possible and has, in the case of Hp, identified 3 SNP which could be genotyped and selectively bred if an association with production traits was found at a later date.

The final study in this thesis investigated a mixed sex pig herd and estimated the heritability of the 4 porcine APP. As previously reported, there were sex differences apparent in Hp and to a lesser extent, in CRP baseline concentrations and as a result, line differences were now found in Hp. Sexual dimorphism in the APP concentrations indicates that APP production may be under hormonal control, most likely through the HPA. This has been substantiated by human studies comparing male and female trauma patients undergoing inflammatory events which shows that females experience lower inflammation (Hsieh *et al.*, 2007).

Heritability estimates were low in all APP especially for TTR. However, the heritability estimate for Hp was similar to a previously reported estimate (Henryon *et al.*, 2006). Although this means that APP baseline concentrations are only a lowly heritable trait this can be overcome by the identification of SNP and associations with phenotype. This would allow genotyping at an early age and selection to occur; in turn, this may improve the heritability estimates and allow a breeding plan to be established.

Breeding for APP concentrations may be useful if, as previously discussed, they are associated with production traits. However, it is also likely that they may become a factor in breeding for resistance to disease. Acute phase proteins can be used as an indication of an organism's state of immunocompetence, it is unknown whether high or low baseline APP concentrations would be more beneficial for an animal. High concentrations may have benefits in protecting the animal from infection but it is possible that in doing so they could divert nutrients from muscle growth whereas low concentrations may not protect the animal effectively. It is also likely that the kinetics of the APR should be investigated in relation to disease resistance and/or inflammatory events such as vaccination and that studies should centre around whether fast or slow responders show associations with their genetic background and/or production traits.

The studies carried out in this thesis were using high health populations of pigs and it is likely that in a conventional environment that there will be changes in heritability estimates and associations with genotype. This will be due to the effect of the environment on APP concentrations which will typically be higher than that which has been reported in this thesis. Indeed this has been reported to be the case in comparisons of SPF and conventional pig units (Petersen *et al.*, 2002b) and which should lead to more variability in APP concentrations and as a result, analysis may reveal additional associations. Studies of APP under both high health and conventional conditions are required in order to assess the effects of environment on APP and potential implications for associations with desirable breeding traits. This is especially important in pig breeding as boars are

commonly kept under SPF/high health conditions whereas their progeny may be in conventional herds. This work could be carried out in a similar fashion to the studies reported in this thesis or alternatively, a population of pigs could be housed under high health conditions for a period of time and APP concentrations measured, then moved into a conventional herd and measured. This would provide a direct comparison and a wealth of information.

In conclusion, this thesis has demonstrated that baseline porcine APP concentrations are affected by different factors including sex and pig line. This may mean that interpreting APP results in a herd situation is difficult but with proper understanding and implementation of routine testing, they have the potential to be used as biomarkers. There is potential for use in breeding decisions as demonstrated by the identification of SNP and their association with serum concentrations and APP baseline concentrations have been identified as heritable traits, albeit at a low level. Additionally, this thesis has demonstrated that further research and understanding of the control mechanisms of APP production in pigs is needed before they potentially become viable biomarkers of porcine health and welfare.

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