The role of defensins in controlling *Salmonella* infection in chickens

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I confirm that the work submitted is my own and that appropriate credit has been given where reference has been made to the work of others.

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A Tous,

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Abstract

Defensins are a family of cysteine-rich vertebrate antimicrobial peptides which can be divided into two main families, α-defensins, found only in mammals, and β-defensins, identified in all vertebrate species. In chickens, only β-defensins have been reported, and are now named avian β-defensins (AvBD). The project aim was to compare AvBDs mRNA levels in different chicken lines considered as susceptible and resistant to different *Salmonella* serotypes. Indeed, a previous study showed that α-defensins expressed in Paneth cells were inhibited after *Salmonella* infection in mice suggesting that the inhibition defensins is a virulence strategy of the intestine pathogen. Therefore, we wished to test the hypothesis that the susceptibility of chicken lines to *Salmonella* infection correlated with decreased AvBD transcript levels, as previously shown in mice.

To date, thirteen AvBDs have been already described in the literature. Here I describe a novel avian β-defensin, named AvBD14. The AvBD14 has two exons and one intron and is only expressed at the mRNA level in the skin and spleen. I also propose that two β-defensins, originally described as gallinacin 1 and 1α which differ by only three amino acids due to three nucleotide substitutions, actually represent polymorphic variants of the same gene, named AvBD1.

Because of their differential expression profiles as previously reported, AvBDs 1 and 2, originally isolated from heterophils, and AvBDs 3, 4 and 5, previously described as peptides expressed by epithelial tissues, were chosen to study their expression in a variety of *in vitro* and *in vivo* systems. In order to develop new bioreagents, I attempted to express AvBDs using the Baculovirus system. Unfortunately, the specific physicochemical characteristics of these antimicrobial peptides made them difficult to purify and they were, therefore, examined by measuring their mRNA expression levels.

In this study, inbred chicken lines 6_1 and N, previously characterized both for their resistance to systemic *Salmonella* disease and their levels of *Salmonella* colonization, were selected to analyze the expression of the AvBD panel chosen. Line 6_1 and line N chickens are resistant and susceptible to *Salmonella* serovar Typhimurium colonisation respectively and, interestingly, mRNAs for AvBDs 2, 3 and 5 were undetectable at 7 dpi in the caecal tonsil of line N chickens infected with *S.* Typhimurium. AvBD1 mRNA expression was also down-regulated soon after infection suggesting that line N susceptibility is a deficiency in innate immunity.

In addition, the differential responses of inbred lines to *Salmonella* serovars indicate the involvement of a common mechanism of resistance. For this purpose, AvBD expression was also analysed in the resistant line, line 6_1, and the susceptible line, line 7_2, following infection with different *Salmonella* serovars. In resistant and susceptible chickens infected with host-specific or broad host range *Salmonella* serovars, mRNA level of AvBDs was differentially expressed, but not inhibited.

In conclusion, we have demonstrated that the level of expression of AvBDs did not determine the resistance or susceptibility pattern of chicken line 6_1 and 7_2 to systemic salmonellosis. However, the expression of several of AvBDs may regulate the resistance of chicken line 6_1 to *S.* Typhimurium colonisation.

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Abbreviations

AITRL	activation-inducible TNF receptor ligand
AMP	antimicrobial peptide
AMV	avian myeloblastosis virus
APRIL	a proliferation-inducing ligand
AvBD	avian β-defensin
BAC	bacterial artificial chromosome
BAFF	B cell activating factor
BNBD	bovine neutrophil β-defensins
BSA	bovine serum albumin
BVDV	bovine viral diarrhoea virus
C1 in	C1 inhibitor
C4BP	C4-binding protein
$CaCl_2$	calcium chloride
CAMP	cationic antimicrobial peptide
CARD	caspase recruitment domain
CBB	Coomassie brillant blue
CD	Crohn's disease
cDNA	complementary DNA
CFU	colony forming unit
CHP	chicken heterophil peptide
CO_2	carbon dioxide
CpG-ODN	oligodeoxynucleotide containing CpG motif
CR1	Complement receptor 1
CSF	colony-stimulating factors
Ct	Cycle threshold
CTBS	Di N-acetylchitobiase
DAF	Decay-accelerating factor
DC	Dendritic cell
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dpi	day(s) post-infection
dsRNA	double-stranded RNA
EBD	enteric β-defensin
EBNA	Epstein-Barr virus-encoded nuclear antigen
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
FASL	Fas ligand
FCS	foetal calf serum
FPLC	fast protein liquid chromatography
g	gram
Gal	gallinacin, chicken β-defensin
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage-colony stimulating factor
HBD	human β-defensin
HCl	hydrochloric acid
HD	human Paneth cell α-defensin

HEK	human embryonic kidney
His	histidine
HNP	human neutrophil α-defensin peptide
HPLC	high performance liquid chromatography
HR	hypersensitive response
HSV	herpes simplex virus
IEC	intestinal epithelial cells
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1R	interleukin-1 receptor
IL-1ra	IL-1 receptor antagonist
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRAK	IL-1R-associated kinase
IRF	interferon-regulatory factor
IκB	inhibitor of nuclear factor-κB
JNK	Jun N-terminal kinase
KCl	potassium chloride
kDa	kilodalton
LAP	lingual antimicrobial peptide
LB	Luria-Bertani
LITAF	LPS-induced TNF-alpha factor
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LT	lymphotoxin
M	mole
M13	bacteriophage M13
Ma	marker
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MASP	MBL-associated serine protease
MAT	gene coding for matrylisin
MBD	mouse β-defensin
MBL	mannan-binding lectin
MbCP	membrane cofactor protein
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony-stimulating factors
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
mM	millimolar
MMP	metalloproteinase matrilysin peptide
$MnCl_2$	manganese chloride
MOP	4-Morpholinepropanesulphonic acid
mRNA	messenger RNA
MW	molecular weight
NaCl	Sodium chloride
NaOH	sodium hydroxide
NEB	New England Biolabs
NF-κB	nuclear factor-κB

NI	non-infected
NK	natural killer
NOD	nucleotide-binding oligomerization domain
OD	optical density
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PAR	protease-activated receptor
pBD	porcine β-defensin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
pI	isoelectric point
pi	post-infection
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PMN	polymorphonuclear cell
PR	pathogenesis-related protein
PRR	pattern recognition receptor
Q-Tof	quadrupole/time-of-flight
RANKL	receptor activator of NF-κB ligand
RbCl	rubidium chloride
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
RP-HPLC	reverse phase-HPLC
RPMI	media developed at Roswell Park Memorial Institute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-PCR
rTth	recombinant *Thermus thermophilus*
S.E.M	standard error of the mean
SAR	systemic acquired resistance system
SBD	sheep β-defensin
SCV	*Salmonella*-containing vacuole
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLPI	secretory leucocyte protease inhibitor
SNP	single nucleotide polymorphism
SOC	super-optimized culture medium
SPF	specific pathogen-free
SPI	*Salmonella* pathogenicity islands
ssRNA	single-stranded RNA
SV40	Simian vacuolating virus 40
TAB	TAK1-binding protein
TAE	Tris-acetate-EDTA
TAK	transforming-growth-factor-β-activated kinase
Taq	*Thermus aquaticus*
TBK	TRAF-family-member-associated NF-κB activator-binding kinase
TFA	trifluoroacetic acid
TGF	transforming growth factor
TIR	Toll/IL-1R

TIRAP	TIR-domain-containing adaptor protein
TL1A	TNF-like ligand 1A
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFSF	TNF superfamily
TRAF	tumour-necrosis-factor-receptor-associated factor
TRAIL	tumor-necrosis-factor related apoptosis inducing ligand
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
TTSS	type III secretion system
TWEAK	TNF-related weak inducer of apoptosis
UC	ulcerative colitis
VEGI	vascular endothelial cell growth inhibitor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1 - Introduction

1.1 Principles of innate immunity and its components

The body, especially the gastrointestinal, respiratory and urogenital tracts, is constantly in contact with commensal bacterial microbiota and can be exposed to infectious organisms. The innate immune response, composed of macrophages, natural killer (NK) cells, neutrophils, mucosal epithelial cells, and endothelial cells, is the first line of defense against a large number of common microorganisms to control potential bacterial infections (Abreu & Arditi, 2004). Innate immunity was long considered as a non-specific response, acting over several days and characterised by engulfment of infectious agents by macrophages. Indeed, microorganisms invading the epithelial surface of the body are usually met by mononuclear phagocytes that recognize and bind conserved and invariant constituents of a class of microorganisms, defined as pathogen-associated molecular patterns (PAMPs). The binding of PAMPs to pattern recognition receptors (PRRs) on cells such as macrophages activates phagocytosis and the secretion of cytokines and chemokines that attracts neutrophils and monocytes, causing local inflammation. By contrast, adaptive immunity, mediated by T and B cells, is a more sophisticated defense mechanism, responding to a specific pathogen antigen and increasing protection against subsequent reinfection by generating memory T and B cells (Akira & Takeda, 2004; Froy, 2005; Hoebe *et al.*, 2004; Williams *et al.*, 2006).

Since the discovery of Toll-like receptors (Medzhitov, 2001), the innate immune response has been considered as the mechanism recognising a particular epitope as "dangerous" or "safe", while the adaptive immune response has been redefined as the mechanism acting against any pathogenic components with a potential to cause host infection (Williams *et al.*, 2006).

1.1.1 Innate cells

The white blood cells called leukocytes can move towards, interact with and engulf microorganisms. Leukocytes derive from a common progenitor, the pluripotential haemopoietic stem cells present in the bone marrow (Alberts, 2002). The division of haemaopoietic stem cells can produce other more specialised types of stem cells including myeloid progenitor stem cells, the precursors of granulocytes, macrophages, dendritic cells and mast cells (reviewed in Janeway, 2005).

Mast cells reside in connective tissue and mucous membranes. They play a role in pathogen defence, wound healing, and once activated, rapidly release granules containing histamine, heparin and other active agents to recruit neutrophils and macrophages. In addition, histamine dilates blood vessels, causing characteristic signs of inflammation including pain, redness, heat and swelling.

Macrophages are large phagocytic leukocytes, highly motile and able to cross the cell membrane of capillary vessels to pursue pathogens. In response to infection, macrophages differentiate from monocytes present in the blood and are activated by PAMPs binding to macrophage surface receptors, triggering phagocytosis and bactericidal mechanisms (reviewed in Janeway, 2005).

Neutrophils belong to the polymorphonuclear (PMN) cell family, also comprising eosinophils and basophils in mammals. By comparison, chicken PMN are composed of heterophils only, which are considered as neutrophil-like cells. Neutrophils represent 50 to 60% of the total circulating leukocytes but they are not present in healthy tissues. Similarly to macrophages, neutrophils recognize, ingest and destroy many pathogens by activating bactericidal mechanisms, without the aid of adaptive immune responses (reviewed in Janeway, 2005).

Dendritic cells (DC) are phagocytic cells present in peripheral tissues such as skin, lungs, stomach and intestines in immature form (Alberts, 2002). The activation of dendritic cell maturation involves the uptake of foreign antigens or stimulation by effector molecules secreted by other phagocytic cells and induces the process of antigen presentation in lymph nodes, thus linking the innate and adaptive immune systems (reviewed in Janeway, 2005).

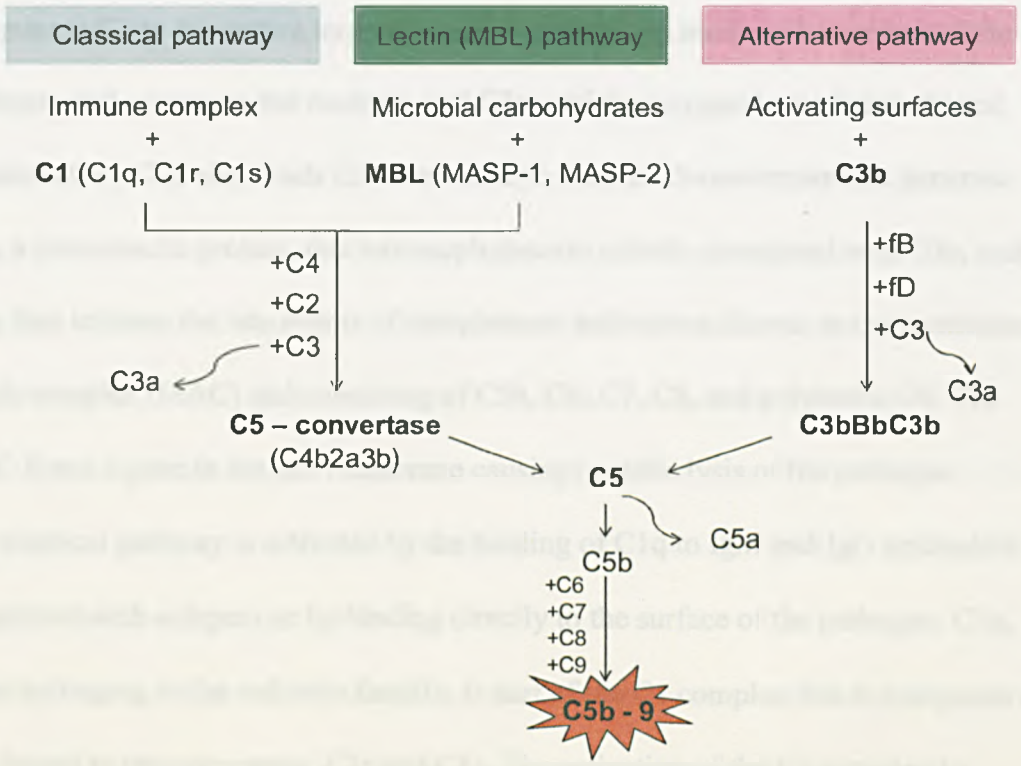
NK cells attack host cells that have been infected by microbes, tumor cells and virally infected cells. The process by which NK cells recognise infected cells is known as "missing-self", as infected cells express abnormally low levels of the cell-surface marker known as the MHC (major histocompatibility complex). NK cells therefore do not require activation to kill cells (reviewed in Janeway, 2005), hence their name.

1.1.2 Innate microbial effector system

The complement system is a biochemical cascade of the immune system consisting of a large number of plasma proteins that helps to fight infection. Many complement proteins are proteases activated by proteolytic cleavage. These enzymes, named zymogens, are widely distributed in body fluids and tissues and their subsequent cleavage induces the enzymatic cascade and amplifies the complement response. The complement system, involved in both innate immunity and acquired immunity, is composed of 35 soluble and cell-bound proteins and its activation leads to cytolysis, chemotaxis, opsonization, immune clearance and inflammation, as well as the marking of pathogens for phagocytosis.

Three biochemical pathways activate the complement system: the classical complement pathway, the alternate complement pathway, and the mannose-binding lectin pathway (Figure 1.1).

Figure 1.1: The complement system, adapted from (Francis *et al.*, 2003). Complement is an innate immune cascade, which is activated by one of the three pathways depending on the nature of the foreign molecule. The classical pathway is activated primarily by the interaction of C1q with immune complexes of antibody with antigen. The alternative pathway leads to the deposition of C3 fragments on the target cells, and the lectin pathway is activated by binding of MBL to carbohydrates expressed on pathogens. The end result of all three pathways is either the opsonisation, through formation of the lytic molecule C5b-9, of target organisms.



These pathways require different molecules for their activation, such as antibodies for activation of the classical complement pathway and protease C3 hydrolysis or antigens for activation of the alternate pathway, but their enzymatic cascades produce the same set of effector molecules, homologous variants of the protease C3-convertase. C3-convertases, bound covalently to the pathogen surfaces, then cleave component C3 to generate a large amount of C3b, which binds to the surface of the pathogen and opsonizes the bacteria, and C3a, which is a peptide mediator of local inflammation. C3b also binds C3-convertase to form a C5-convertase that generate C5a, a chemotactic protein, that has anaphylatoxin activity associated with C3a, and C5b, that initiates the late events of complement activitation known as the membrane attack complex (MAC) and consisting of C5b, C6, C7, C8, and polymeric C9. The MAC forms a pore in the cell membrane causing osmotic lysis of the pathogen.

The classical pathway is activated by the binding of C1q to IgM and IgG antibodies complexed with antigens or by binding directly to the surface of the pathogen. C1q, a lectin belonging to the collectin familly, is part of the C1 complex that is composed of C1q bound to two zymogens, C1r and C1s. The activation of the C1 complex by binding of C1q leads to conformational changes in the $(C1r:C1s)_2$ complex causing the activation of two C1r molecules, which are serine proteases. The active form of C1r cleaves C1s to generate another active serine protease. C1s then binds to and splits C2 and C4 to produce two large fragments, C2a and C4b, which together form the C3-convertase of the classical pathway. C4b binds covalently to the pathogen surface to form a C3-convertase that remains on the surface of the pathogen once complexed with the active serine protease, C2b, which cleaves C3 molecules.

The mannan-binding lectin pathway is activated by mannan-binding lectin, a serum protein, binding to mannose residues on the pathogen surface. The mannan-binding

lectin is a six-headed molecule that forms a complex with two proteases, MASP-1 and MASP-2. Binding of the MBL-associated serine proteases to the pathogen surface initiates the cleavage of C4 and C2 by MASP-1 and MASP-2 into C4b and C2a. C4b and C2a then bind together to form C3-convertase and activate the complement cascade in the same way as the classical pathway.

The alternative pathway is triggered by C3 hydrolysis producing C3b molecules that coat the pathogen surface. The C3 protein is produced in the liver and C3b is produced by spontaneous cleavage. However, in the absence of pathogen C3b is inactivated. Once C3b coats the pathogen surface, it binds to factor B forming a complex, which is then cleaved by factor D into Ba and the alternative pathway C3-convertase, Bb. Some pathogens express complement-regulatory proteins on their surface that bind the C3b,Bb complex, remove Bb and inactivate C3b. If the bacterial surface does not express complement-regulatory proteins, factor P binds and stabilizes the C3b,Bb convertase activity.

The regulation of the complement system involves complement control proteins to protect the host against the complement components, which can be extremely damaging for the host. The complement control proteins are present at a higher concentration in the blood plasma than the complement proteins and act on different stages of the complement cascade. For example, C1 inhibitor dissociates C1r and C1s from the active C1 complex in the classical pathway and CD59 inhibits C9 polymerisation and prevents the final assembly of the MAC complex (reviewed by Janeway, 2005).

In chickens, all the components of the classical and alternative pathway except C2 were first detected in the serum (Barta & Hubbert, 1978). Evolutionary studies confirmed the lack of C2 and suggested that chicken factor B-like protease could be a

common ancestral protein for mammalian complement components B and C2 (Kjalke *et al.*, 1993). However, unpublished observations by K. Skjoedt and J. Kaufman identified homologues of both the C2 and factor B genes in the chicken genome (Lynch *et al.*, 2005). In contrast, C9, which is a terminal complement component in mammals, was not identified in the chicken draft genome (Nonaka & Kimura, 2006). Chicken complement components of the lectin pathway were also described (Lynch *et al.*, 2005). There is a single MBL gene in the chicken, while mammals have two different MBL forms (Laursen *et al.*, 1998), and a MBL-MASP complex, constisting of MASP-2 and MASP-3 only (Lynch *et al.*, 2005). The avian lectin pathway is deficient in MASP-1 but the absence of the protein did not affect the activation of C4 (e.g. Table 1.1).

1.1.3 Innate microbial sensor molecules

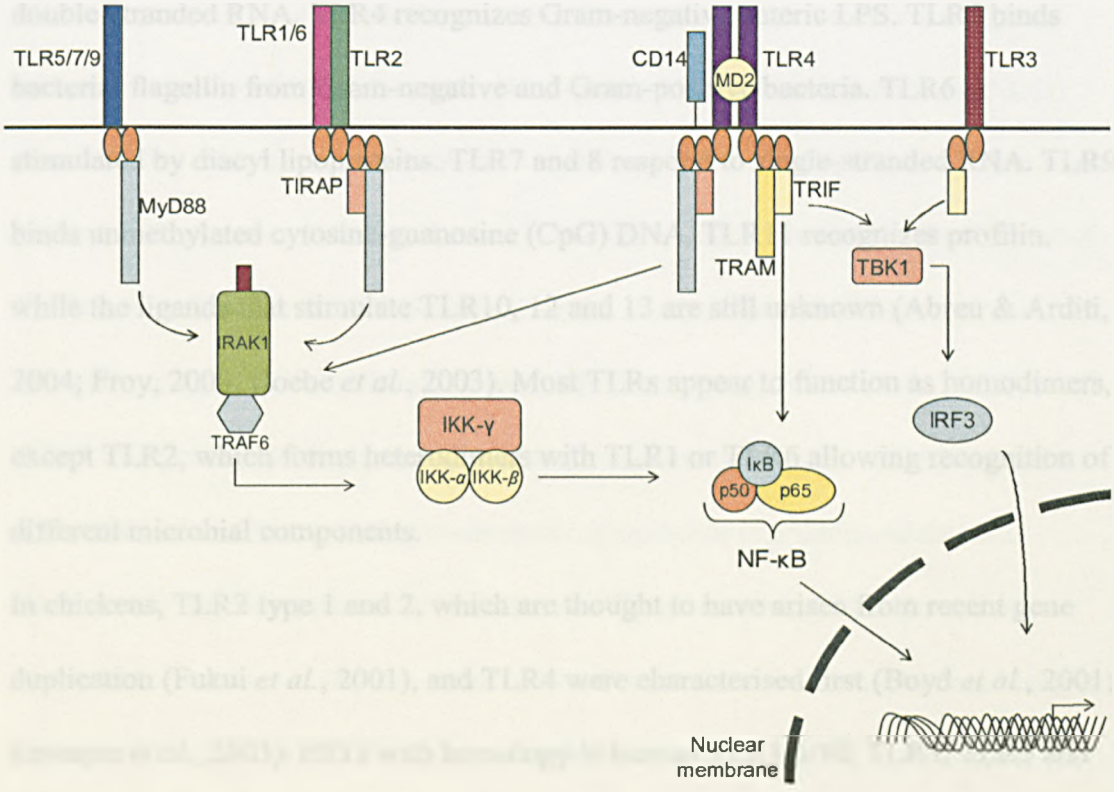
Recognition of pathogenic microbes in mammalian mucosa is mainly conferred by membrane-bound Toll-like receptors. The receptors were named Toll-like because of their similarity with a receptor from the Toll family firstly identified in the fruit fly *Drosophila melanogaster*. *Toll* plays an essential role in the innate immune response against fungal infection (Lemaitre *et al.*, 1996) and has homology with the interleukin-1 receptor (IL-1R) in mammals (Gay & Keith, 1991). The identification of TLRs in human was first described by Nomura *et al.* (1994) and their functions were finally discovered in 1998 with the identification of TLR4 in mice responding to LPS (Poltorak *et al.*, 1998). These transmembrane proteins are composed of a cytoplasmic region with a similar structure to the interleukin 1 receptors and an external region composed of leucine-rich repeat (LRR) motifs. The conserved cytoplasmic region, known as the Toll/IL-1R (TIR) domain, is composed

Table 1.1: Comparison of complement component genes in different groups of vertebrates (adapted from Nonaka and Kimura, 2006). +, presence of the component in the animal group; -, absence of the component in the animal group; ND, presence/absence has not been determined.

	Mammalia	Aves/Reptilia	Amphibia
C3	+	+	+
C4	+	+	+
C5	+	+	+
Factor B	+	+	+
C2	+	+	+
C1q	Encoded by 3 genes	Encoded by a single gene	+
MBL	Encoded by 2 genes	Encoded by a single gene	+
MASP-1	+	-	+
MASP-2	+	+	+
MASP-3	+	+	+
C1r	+	+	+
C1s	+	+	+
C6	+	+	+
C7	+	+	+
C8a	+	+	+
C8b	+	+	+
C9	+	-	+
Factor D	+	ND	ND
Factor I	+	+	+
Factor H	+	+	+
C4BP	+	+	+
DAF	+	+	+
MbCP	+	ND	ND
CR1	+	ND	ND
CR2	+	ND	ND
Integrin aM	+	+	+
Integrin aX	+	+	+
Integrin b2	+	+	+
Properdin	+	-	+
C3a R	+	+	+
C5a R	+	+	+
C1 in	+	+	-
CD59	+	+	+
Clusterin	+	+	+
Factor S	+	+	+

of three conserved boxes essential for signalling, named the R, A and S faces. The R and A interface would mediate the oligomerization of receptor TIR domains in response to the association of a ligand with the TLR to facilitate the interaction of downstream adapter molecules such as MyD88 with the TIR domain. The third interface, the S face, would mediate the association between the receptor and adapter TIR domains (Xu *et al.*, 2000). Stimulation of TLRs triggers complex signalling pathways (Figure 1.2), either MyD88-dependent or -independent, allowing translocation to the nucleus of transcription factors including nuclear factor-κB (NF-κB), to induce the expression of target genes, such as tumour necrosis factor (TNF)-α, pro-inflammatory cytokines and antimicrobial peptides (Akira & Takeda, 2004). MyD88 is an intracellular adaptor molecule that binds the TIR domain and recruits IRAK4 (IL-1R-associated kinase), IRAK1and TRAF6 (tumour-necrosis-factor-receptor-associated factor). Phosphorylated IRAK1 and TRAF6 dissociate from the TIR domain to form a complex with TAK1 (transforming-growth-factor-β-activated kinase) and two binding proteins, TAB1 and TAB2. Once in the cytosol, TRAF6 is ubiquitylated and triggers the activation of TAK1, which in turns activates the IκB kinase kinase complex involved in the activation of transcription factors including NF-κB (Akira & Takeda, 2004; Froy, 2005). By contrast, the MyD88-independent pathway involves interferon-regulatory factor 3 (IRF3). The TIR domain interacts with the terminal region of TRIF (TIR-domain-containing adaptor protein inducing IFN-β) that binds both TRAF6 and TBK1. TBK1, activated by TRIF, phosphorylates IRF3 that mediates the initial induction of type I IFNs, which in turn activate the expression of IFN-inducible genes. In parallel, TRAF6 mediates the activation of the NF-κB (Akira & Takeda, 2004). MyD88 is therefore an adaptor molecule that activates other molecules within the cell. Three other adapter molecules are also

Figure 1.2: MyD88-dependent and independent pathways of different TLRs (Akira & Takeda, 2004). The TIR-domain-containing adaptor molecule MyD88 mediates the Toll-like receptor signalling pathway. TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 use this pathway that releases NF-κB from its inhibitor so that it translocates to the nucleus and induces expression of inflammatory cytokines. TIRAP-domain is also involved in the MyD88-dependent signalling pathway through TLR2 and TLR4. By contrast, TLR3 and TLR4 activate the interferon IRF3 in a MyD88-independent manner. In addition, TLR3 use a third TIR-domain-containing adaptor, TRIF, which is essential for the MyD88-independent pathway. While TLR4 use a fourth TIR-domain-containing adaptor, TRAM, which is specific to the MyD88-independent/TRIF-dependent pathway.



involved in the MyD88-independent pathway, including TIRAP, TRIF and TRAM. Each TLR uses one or multiple adapters according to the type of stimulation by different ligands. To date, thirteen TLRs, TLR1 to TLR13, have been identified in humans and mice together (Du *et al.*, 2000). TLRs 11, 12, and 13 are expressed in mice but they have not been found in human. TLRs are stimulated by different ligands. TLR1 is stimulated by triacyl lipoproteins. TLR2 responds to a variety of ligands including peptidoglycan, lipopeptides and lipoproteins. TLR3 is stimulated by double-stranded RNA. TLR4 recognizes Gram-negative enteric LPS. TLR5 binds bacterial flagellin from Gram-negative and Gram-positive bacteria. TLR6 is stimulated by diacyl lipoproteins. TLR7 and 8 respond to single-stranded RNA. TLR9 binds unmethylated cytosine-guanosine (CpG) DNA. TLR11 recognizes profilin, while the ligands that stimulate TLR10, 12 and 13 are still unknown (Abreu & Arditi, 2004; Froy, 2005; Hoebe *et al.*, 2003). Most TLRs appear to function as homodimers, except TLR2, which forms heterodimers with TLR1 or TLR6 allowing recognition of different microbial components.

In chickens, TLR2 type 1 and 2, which are thought to have arisen from recent gene duplication (Fukui *et al.*, 2001), and TLR4 were characterised first (Boyd *et al.*, 2001; Leveque *et al.*, 2003). ESTs with homology to human TLR1/6/10, TLR3, TLR5 and TLR7 were then identified (Iqbal *et al.*, 2005; Lynn *et al.*, 2003). Chicken TLR8 was also identified but sequence analysis showed that the chTLR8 gene was disrupted by 6-kilobase insertion containing chicken repeat 1 retroviral-like insertion elements (Philbin *et al.*, 2005). In addition to the identification of various chicken TLRs, MyD88, TIRAP, IRAK-1, IRAK-2, IRAK-4 and NF-κB signalling components were also characterised (Smith *et al.*, 2004). All the chicken TLRs were shown to be expressed at the mRNA level in various chicken tissues (Iqbal *et al.*, 2005) and

heterophils (Kogut *et al.*, 2005) and showed to be functional in response to different ligands (e.g. Table 1.2). Chicken cells respond to CpG-ODN, but analysis of the chicken genome failed to identify TLR9 (He *et al.*, 2003; He *et al.*, 2007; Xie *et al.*, 2003). Recently, two novel TLRs were identified in chicken; TLR21 (Roach *et al.*, 2005) and TLR15, which was expressed in the spleen, bursa, and bone marrow of healthy chickens and up-regulated in the caeca of chickens infected with *S.* Typhimurium (Higgs *et al.*, 2006).

TLR-independent systems also have roles in the recognition of certain pathogens, such as the family of cytosolic nucleotide-binding oligomerization domain (NOD) proteins. The NOD system contains two proteins, NOD1 and NOD2, composed of an N-terminal caspase recruitment domain (CARD), a central nucleotide-binding domain (NOD) and a C-terminal LRR domain (Inohara *et al.*, 2005). NOD2 recognizes bacterial peptidoglycan by detecting dipeptide muramyl (Girardin *et al.*, 2003), while NOD1 recognize peptidoglycans containing mesodiaminopimelic acid (Chamaillard *et al.*, 2003). In addition, these sensor proteins modulate expression of cationic antimicrobial peptides, particularly defensins (Boughan *et al.*, 2006; Voss *et al.*, 2006), and mutations in these proteins affected defensin expression and could trigger the development of intestinal inflammatory diseases (Wehkamp *et al.*, 2004b).

To date, a sequence in the chicken genome, LOC420677, was identified to be similar to NOD1 and located on chromosome 2.

1.1.4 Innate microbial effector molecules

In response to microbial infection, the innate immune system secretes a range of effector molecules with different functions, such as cytokines, alarm proteases and antimicrobial peptides (AMPs).

Table 1.2 Summary of known mammalian and chicken TLRs, ligands and adapters. ***TLR1/6/10 are used in mammals as homodimers or heterodimers. In chicken, 2 TLRs were identified to be homologous to human TLR1/6/10 group.**

Receptor	Ligand(s)	Adapter(s)	In the chicken
TLR1	triacyl lipoproteins	MyD88/TIRAP	**TLR1/6/10**, 2 genes (Iqbal *et al.*, 2005; Lynn *et al.*, 2003)*
TLR2	lipoproteins; gram positive peptidoglycan; lipoteichoic acids;	MyD88/TIRAP	**TLR2 type 1 and 2**, 2 genes (Fukui *et al.*, 2001)
TLR3	dsRNA, poly I:C	TRIF	**TLR3** (Iqbal *et al.*, 2005; Lynn *et al.*, 2003)
TLR4	lipopolysaccharide; viral glycoproteins	MyD88/TIRAP/TRIF/TRAM	**TLR4** (Boyd *et al.*, 2001; Leveque *et al.*, 2003)
TLR5	flagellin	MyD88	**TLR5** (Iqbal *et al.*, 2005; Lynn *et al.*, 2003)
TLR6	diacyl lipoproteins	MyD88/TIRAP	See above
TLR7	synthetic compounds; ssRNA	MyD88	**TLR7** (Philbin *et al.*, 2005)
TLR8	synthetic compounds; ssRNA	MyD88	**TLR8**, disrupted (Philbin *et al.*, 2005)
TLR9	unmethylated CpG DNA	MyD88	No
TLR10	unknown	unknown	See above
TLR11	Profilin	MyD88	No
TLR12	unknown	unknown	No
TLR13	unknown	unknown	No
TLR15	unknown	unknown	**TLR15** (Higgs *et al.*, 2006)
TLR21	unknown	unknown	**TLR21** (Roach *et al.*, 2005)

Cytokines are a group of small water-soluble proteins and glycoproteins of 8-30 kDa involved in both innate and adaptive immune responses. They are secreted by many types of cells in response to immunological, inflammatory and infectious diseases and act by binding to specific cell-surface receptors. The action of cytokines can be autocrine, if the cytokine acts on the cell that secretes it; paracrine, affecting the behaviour of another cell; or endocrine, affecting the behaviour of cells distant in the body. The binding of cytokines to specific receptors induces an intracellular signalling response that upregulates or downregulates several inflammatory genes in response to pathogen infection. Cytokines and their receptors can be classified in three main families: firstly, the haematopoietin family composed of the interleukin and the IFN subfamilies; secondly, the TNF family; finally, the chemokine family which can be subdivided into pro-inflammatory chemokines, induced by the immune response, and homeostatic chemokines. They can be released by many different types of cells and recruit leukocytes to the site of infection. Chemokines are structurally divided into two main groups - CC chemokines (or β-chemokines) with two adjacent cysteines near the amino terminus of the protein, and CXC chemokines (or α-chemokines) in which the two cysteines are separated by any amino acid other than proline or cysteine. The CC chemokines bind to CC chemokine receptors (CCR), with ten CCR identified to date in mammals, and induce the migration of monocytes and other cell types such as NK cells and dendritic cells. The CXC chemokines bind to CXCRs, with seven indentified to date in mammals, and induce the migration of neutrophils (reviewed by Janeway, 2005).

In the chicken, the immune system is different to that of mammals and lacks a number of organs, cells and molecules identified in mammals, such as functional eosinophils, IgE and lymph nodes, suggesting a reduce cytokine repertoire. For example, the IL-1

family in human include IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1ra) located on chromosome 2 with six other IL-1 family members, IL-1F5-10 (Dunn *et al.*, 2001), IL-18 (Bazan *et al.*, 1996) that lies on chromosome 11 (Nolan *et al.*, 1998), and IL-33 (IL-1F11) that mediates its biological effects via the IL-1 receptor and lies on chromosome 9 (Schmitz *et al.*, 2005). Only two IL-1 family members have been cloned in the chicken; IL-1β (Weining *et al.*, 1998), encoded on chromosome 4 (Kaiser *et al.*, 2004) and IL-18 (Schneider *et al.*, 2000). The IL-10 family, composed of IL-10, IL-19, IL-20 and IL-24 on chromosome 1 and IL-22 and IL-26 on chromosome 12 in human (Fickenscher *et al.*, 2002), is also reduced in the chicken with four members; IL-10 and IL-19 on chromosome 26, and IL-22 and IL-26 on chromosome 1 (Rothwell *et al.*, 2004). Similarly, the ChIL-17 family is composed of four members - IL-17A, IL-17B, IL-17C and IL-17F (Kaiser *et al.*, 2005), while the human IL-17 family contains six members, IL-17A-F (Moseley *et al.*, 2003). In contrast, interleukins involved in Th1 responses, such as IL-23 and IL-27, were not identified in chickens (Kaiser *et al.*, 2005), while the Th2 interleukins, IL-3, IL-4, IL-5, IL-13 and GM-CSF in mammals (Avery *et al.*, 2004) have an extra family member in the chicken, KK34 (Koskela *et al.*, 2004). The type I interferons, originally discovered to interfere with viral replication, include IFN-α, IFN-β and IFN- λ, which have been identified in the chicken as well (Kaiser *et al.*, 2005). However, the chicken has at least nine IFN-α genes and one IFN-β gene, located on chromosome Z (Kaiser *et al.*, 2005; Sick *et al.*, 1996), while the human genome contains thirteen IFN-α genes and one IFN-β. A single copy IFN-λ gene is encoded on chromosome 7 in the chicken (Kaiser *et al.*, 2005), while three IFN-λ genes are found in the equivalent single locus on human chromosome 19 (Kempuraj *et al.*, 2004). To date, chicken IFN-α and IFN-β have been shown to have antiviral activity (Sick *et al.*, 1996), while the biological

activity of IFN- λ remains to be determined. Other cytokines, including transforming growth factors (TGF) and colony-stimulating factors (CSF), have also been identified in the chicken. The CSF family is involved in the development of myeloid cells, and includes granulocyte-macrophage (GM)-CSF, granulocyte (G)-CSF and macrophage (M)-CSF in mammals. Chicken GM-CSF was identified on chicken chromosome 13 (Avery *et al.*, 2004) and the chicken myelomonocytic growth factor is the chicken G-CSF orthologue (Santos *et al.*, 2006). The TGF-β family contains three genes in chickens, as in mammals. Chicken TGF-β are TGF-β2, TGF-β3 and TGF-β4 and in mammals the TGF-β family contains TGF-β1-3 (Kaiser *et al.*, 2005). Mammalian TGF-β1 and chicken TGF-β4 are orthologues with anti-inflammatory properties (Pan & Halper, 2003).

The second class of cytokines, the tumor necrosis factor (TNF) superfamily, is composed of 17 genes in mammals. Some of them have been identified in chickens, including CD40L, CD30L, VEGI, TRAIL, RANKL, FASL, OX40 and BAFF. The AITRL orthologue in the chicken has weak percentage identity with the mammalian gene, but TNF-α, lymphotoxin (LT)-α, LT-β, 4-IBBL, CD27L, LIGHT, TWEAK and APRIL were not identified in the chicken genome (Kaiser *et al.*, 2005). Despite chicken TNF-α not being identified to date, characterisation of the chicken homologue of LPS-induced TNF-α factor (LITAF) allowed the expression of TL1A (TNFSF 15) *in vitro*, suggesting that chicken LITAF may play a role in the regulation of expression of TNF family members (Hong *et al.*, 2006). In addition, potential receptors for 4-IBB and TNF-α were also identified, suggesting that TNF-α and 4-IBB may be present and that more investigations are needed (Kaiser *et al.*, 2005). However, the receptors for six other members of the TNF family were not identified, suggesting that these TNF superfamily genes are definitely absent from the chicken

genome. Interestingly, a novel TNF member was identified in chicken, TRAIL-L, but this member lacks an apparent receptor and a mammalian orthologue (Kaiser *et al.*, 2005).

Finally, the chemokine repertoire in chicken is also reduced with fourteen CC ligands, of which four are in the macrophage inflammatory protein (MIP) family and six in the monocyte chemoattractant protein (MCP) family. The chicken MIP and MCP families lie on chromosome 19 in two clusters and correspond to two clusters of genes on human chromosome 17, with nine members in MIP family and six in the MCP family (Hughes *et al.*, 2007). Four other CC ligands were identified in chickens with clear orthologous relationships to mammalian CC ligands, with CCL17 and CCL20 located on chromosome 11 and chromosome 9 respectively, and CCL19 and CCL21 located on the chicken sex chromosome Z. Interestingly, orthologues of CCL11, CCL24 and CCL26, which are required for eosinophil and basophil attraction, are missing in the chicken genome, matching with the lack of functional eosinophils (Kaiser *et al.*, 2005). The CXC ligands which play a role in inflammation in human, CXCL1-8, are located on chromosome 4 (Moser *et al.*, 2004). The chicken contains in the equivalent region of the genome three CXCL genes, with two of them encoding the previously described CAF and K60 (Sick *et al.*, 2000). Chicken chromosome 4 also contains three adjacent genes that cluster with mammalian CXCL13, a homeostatic CXCL B cell chemoattractant. Two other homeostatic CXCL chemokines were identified as chicken orthologues of mammalian CXCL12 and CXCL14, located on chromosomes 6 and 13 respectively. The chicken genome also contains single copy genes for both XC ligand and CX3C ligand, five CC receptors and three CXC receptors (Kaiser *et al.*, 2005).

Phylogenetic analysis of chicken cytokine genes, compared to human and mouse cytokine genes, showed that some cytokines, such as IL-1, IL-19 and IFN-λ, were in higher numbers in the mammalian genome, presumably because of gene duplication events. In comparison, the relationship between the relative small number of chemokines in chicken genome, particularly the CC ligands, and the mammalian chemokines is still not clear after phylogenetic analysis (Kaiser *et al.*, 2005).

Alarm Proteases, which are secreted by the host, limit tissue damage after an inflammatory response. Indeed, the degradation of ingested pathogens by phagocytic inflammatory cells triggers the secretion of proteases that degrade the tissues (Dallegri & Ottonello, 1997). In order to limit tissue damage, the host then secretes antiprotease proteins that neutralize any excess protease expression and protect the epithelial barrier. Antiprotease proteins are classified in two groups, the "systemic" and the "alarm" antiprotease proteins. The systemic antiproteases, including α1-proteinase inhibitor and α1-antichymotrypsin, are mostly secreted by hepatocytes, while the alarm antiproteases, including secretory leucocyte protease inhibitor (SLPI) and elafin, are synthesised by epithelial cells at the inflammation site (Sallenave, 2000). SLPI and elafin are small peptides, 11.7 kDa and 9.9 kDa respectively, and members of the four-disulphide core family. However, their antiprotease activity was not the only bioactivity identified. Alarm antiproteases also have antimicrobial activity, anti-inflammatory activity, tissue remodelling activity and exhibit influence on adaptive immunity as well as AMPs (Williams *et al.*, 2006). In the chicken, the presence of alarm proteases has yet to be determined.

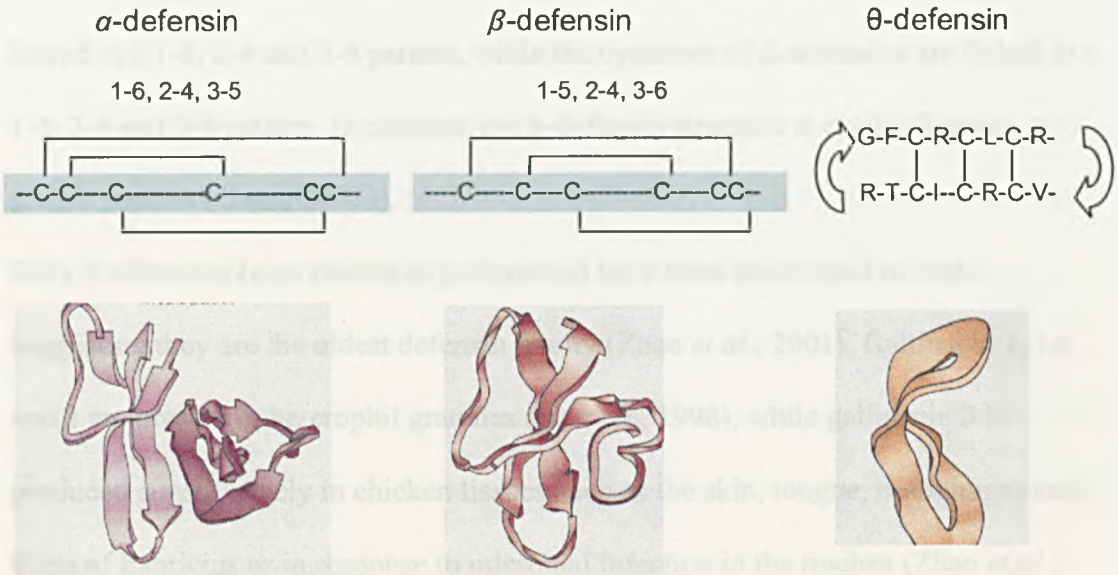
AMPs, including cathelicidins, lysozyme and defensins, are different in structure and their regulation is still under study but they all show additional functions to their antimicrobial activities. Cathelicidins are expressed as precursor peptides,

approximately 18 kDa in size, containing an NH_2-terminal signal peptide, a pro-peptide domain and a variant COOH-terminal antimicrobial functional domain encoding a mature peptide between 12 and 80 amino acids long that gives a bioactive peptide with variant bactericidal potential. In human, the mature peptide, LL-37, was identified in myeloid cell granules, skin and respiratory epithelia and bound LPS to neutralize its endotoxin activity, was chemotactic for neutrophils, monocytes and mast cells and activated denditric cell differentiation, in addition to its antimicrobial activity against gram-negative and gram-positive bacteria. Similarly, lysozyme is a multifunctional protein of 14.3 kDa involved in the activation of the immune system by interacting with antigen-presenting cells and stimulating phagocytic activity of neutrophils and macrophages. First identified as an ancient enzyme, lysozyme contains four cysteines forming disulphide bridges and is expressed by circulating leukocytes and certain tissues such as gastric and pyloric glands, Bruner's glands and Paneth cells. The third group of AMPs, defensins, are to date the most abundant and the best characterised family. In human, six α-defensins including four neutrophil α-defensins and two intestinal α-defensins have been identified so far (Dommett *et al.*, 2005) and thirty nine β-defensins genes and pseudogenes have been discovered (Patil *et al.*, 2005) but to date only four β-defensins have been intensively studied with some of them showing differential regulation in response to pathogen infection.

1.2 Defensins

Defensins are a family of vertebrate antimicrobial peptides. The two main subfamilies of defensins are α- and β-defensins, with an additional distinct subfamily identified in rhesus macaque monkey leukocytes, the θ-defensins (Figure 1.3). The α- and β-defensins consist of a triple-stranded β-sheet and a framework of six

Figure 1.3: Defensin peptides. Numbers above the disulphide schemes indicate the disulphide connections (adapted from Selsted and Ouellette, 2005). The three-dimensional structures are the human neutrophil 3 dimer, an α-defensin, human β-defensin 2 and θ-defensin RTD-1 (Ganz, 2003a) respectively.



disulphide-linked cysteines (Ganz, 2003a). The α-defensins are 29-35 amino acids in length, while the β-defensins are longer and consist of 38-42 amino acids (Raj & Dentino, 2002). The α- and β-defensins differ both in the length of peptide segments between cysteines and the pairing of cysteines: the cysteines of the α-defensins are linked in a 1-6, 2-4 and 3-5 pattern, while the cysteines of β-defensins are linked in a 1-5, 2-4 and 3-6 pattern. In contrast, the θ-defensin structure is cyclic, forming a simple β-sheet (Ganz, 2003a).

Only β-defensins (also known as gallinacins) have been discovered in birds, suggesting they are the oldest defensin family (Zhao *et al.*, 2001). Gallinacin 1, 1α and 2 are present in heterophil granules (Harmon, 1998), while gallinacin 3 is produced constitutively in chicken tissues such as the skin, tongue, oesophagus and bursa of Fabricius or in response to microbial infection in the trachea (Zhao *et al.*, 2001). More recently, ten more sequences coding for defensins were identified in the chicken genome (Lynn *et al.*, 2004; Xiao *et al.*, 2004), all thirteen gallinacin genes being encoded on chromosome 3. Similarly to the human and mice defensin loci, the *CTBS* gene, coding for Cathepsin B, and a human EST sequence (accession no. BE072524) were localised on either side of the chicken defensin cluster. These conserved genes were first identified in defensin gene clusters on human chromosome 8p22 and mouse chromosome 14C3 (Xiao *et al.*, 2004). Phylogenetic analysis of vertebrate β-defensins showed that the bootstrap support was very weak for the clustering of chicken β-defensins with mammalian homologues, suggesting that the β-defensin family arose before the divergence of birds and mammals, and α-defensins evolved after the divergence of mammals from other vertebrates.

Defensin-like antibacterial peptides have also been identified in plants and insects, with a characteristic four-disulphide linkage, and designated as γ-thionins (Raj &

Dentino, 2002). Such defensins have not been yet found in vertebrates. Defensins with six cysteines in disulphide linkages have also been identified in plants and insects (Ganz, 2003a). Insect defensins include phormicins, sapecins, royalisin and spodoptericin and they appear to be secreted by different insect species such as Diptera, Coleoptera, Hymenoptera, Hemiptera and Lepidoptera. Insect defensins showed antimicrobial activity against Gram-positive bacteria and filamentous fungi and, similarly to mammalian defensins, the killing mechanism involves interaction of the peptide with the pathogen surface, disrupting the permeability barrier of the cytoplasmic membrane and killing the microorganism. Insect defensins are small cationic peptides synthesized as precursor propeptides and, interestingly, they contain six or eight cysteine residues, which stabilize the defensin structures by formation of disulphide bridges and increase the variety of insect defensin structural features, which can be classified in three groups including $\alpha\beta\beta$ or $\beta\alpha\beta\beta$ defensins, triple-stranded antiparallel β-sheet defensins and hairpin-like β-sheet structure defensins (Dassanayake *et al.*, 2007). Defensins have also been identified in a variety of plants, including wheat, barley, spinach, pea, and several members of the Brassicaceae family, inhibiting the growth of fungi without being toxic to either mammalian or plant cells. The first plant defensins identified were Rs-AFP1 and Rs-AFP2, isoform peptides isolated from radish seeds belonging to the Brassicaceae family (Solis *et al.*, 2006). Because plants lack an adaptive immune system, they evolved an antimicrobial defence mechanism that includes the production of pathogenesis-related (PR) proteins by the hypersensitive response (HR) or the systemic acquired resistance (SAR) system induced by pathogen infection. Eleven PR protein families were first discovered (Fritig *et al.*, 1998), then three more PR protein families were included such as the plant defensin family PR-12 (van Wees *et al.*, 1999). A recent genome

analysis of *Arabidopsis thaliana* revealed 317 homologous defensin-like sequences and that a subset of these defensins was expressed. Therefore, defensins may have evolved into such a large multigene family in order to provide non-host resistance, which is a phenomenon where the entire plant is resistant to a specific pathogen, providing durable protection in the field. In addition, plant defensins seem also to be involved in the protection of symbiotic bacteria such as *Rhizobium*. Indeed, the nodule, which is nutrient-rich, can attract many pathogens. However, some defensins are expressed in the nodule of the *Medicago truncatula* plant, suggesting that the antimicrobial peptides were secreted to protect the nutrient-rich environment (Silverstein *et al.*, 2005).

Therefore, because of the variety of multicellular organisms, from the vegetable to the animal kingdom, capable of producing defensin peptides, the antimicrobial peptides can be considered as an ancient first line of host defence against pathogen infection.

1.2.1 Cellular distribution

Defensins are synthesised in granulocytes or secreted by epithelial cells and contribute to host defence against microbial colonisation and infection. Leukocytes, such as neutrophils and macrophages in mammals and heterophils in poultry, are components of the innate immune system. They produce either α- or β-defensins: avian heterophils produce β-defensins, human neutrophils synthesise α-defensins, while human monocytes, macrophages and dendritic cells secrete β-defensins (Duits *et al.*, 2002) and mice neutrophils do not express any defensins (Table 1.3). Epithelial cells also secrete defensins either constitutively or in response to infection, acting as an important antimicrobial barrier as part of innate immunity. They may also act as important effector cells of the adaptive immune system (Kamal *et al.*, 2001). One of

Table 1.3: Diverse patterns of α- and β-defensin expression in human, mice and chicken (Ganz, 2003a).

Species	Neutrophils/ heterophils	Paneth cells	Epithelial tissues
Human	α-defensins	α-defensins	α- & β-defensins
Mouse	none	α-defensins	α- & β-defensins
Chicken	β-defensins	not determined[1]	β-defensins

[1]It is not clear if chickens possess Paneth cells.

the epithelial cell types involved in the primary defence of the small intestine in mammals is the Paneth cell. These cells are located at the base of the intestinal crypt and are generated by stem cells, which produce intestinal epithelial cells, including goblet cells, which produce mucin, the absorptive enterocytes and enteroendocrine cells. Paneth cells differentiate as they migrate down to the crypt base, while the three other cell types migrate from the crypt to the villus tip. However, Paneth cells have not been described in the chicken yet. The protective role of Paneth cells may explain the difference in their migration pattern and their ability to secrete defensins. These cells tend to secrete α-defensins in response to pathogen infection. Other epithelial tissues secrete mostly β-defensins in mammals, including the tongue epithelium in pigs and sheep (Ganz, 2003a; Meyerholz *et al.*, 2004) or trachea in the cow and sheep (Ganz, 2003a; Meyerholz *et al.*, 2004), suggesting a generalised protective role for defensins for mucosal surfaces as part of the innate immune system.

1.2.2 Expression and maturation

Defensins are expressed either constitutively or in response to pathogen infection. The α-defensins are generally synthesised and stored in granules of mature leukocytes. They are also synthesised by Paneth cells in an inactive form (Raj & Dentino, 2002). The immature defensins consist of a tripartite prepropeptide with a precursor sequence of 90-100 amino acids containing an amino (NH_2)-terminal signal sequence of about 19 amino acids, an anionic propiece of about 45 amino acids and a carboxy (COOH)-terminal mature cationic defensin of about 30 amino acids. The negative charge of the propiece usually neutralises the positive charge of the mature defensin preventing the premature interaction of defensins with the membrane of neutrophils and Paneth cells. The process of maturation involves enzymes such as the

metalloproteinase, matrilysin, in mice (Wilson *et al.*, 1999) or three forms of trypsin in human (Ganz, 2003a).

The avian β-defensins stored in heterophil granules consist also of tripartite prepropeptide sequences with a precursor sequence containing an NH_2-terminal signal sequence, a basic or neutral propiece and a mature cationic defensin of about 40 amino acids. However, the avian β-defensin propeptide is probably unable to neutralise the mature peptide. For example, gallinacin 1 is not negatively charged whereas gallinacin 2 has only one negative charge. Therefore, some other mechanism must be involved in neutralising premature activity of the chicken β-defensin (Brockus *et al.*, 1998).

1.2.3 Defensin activities

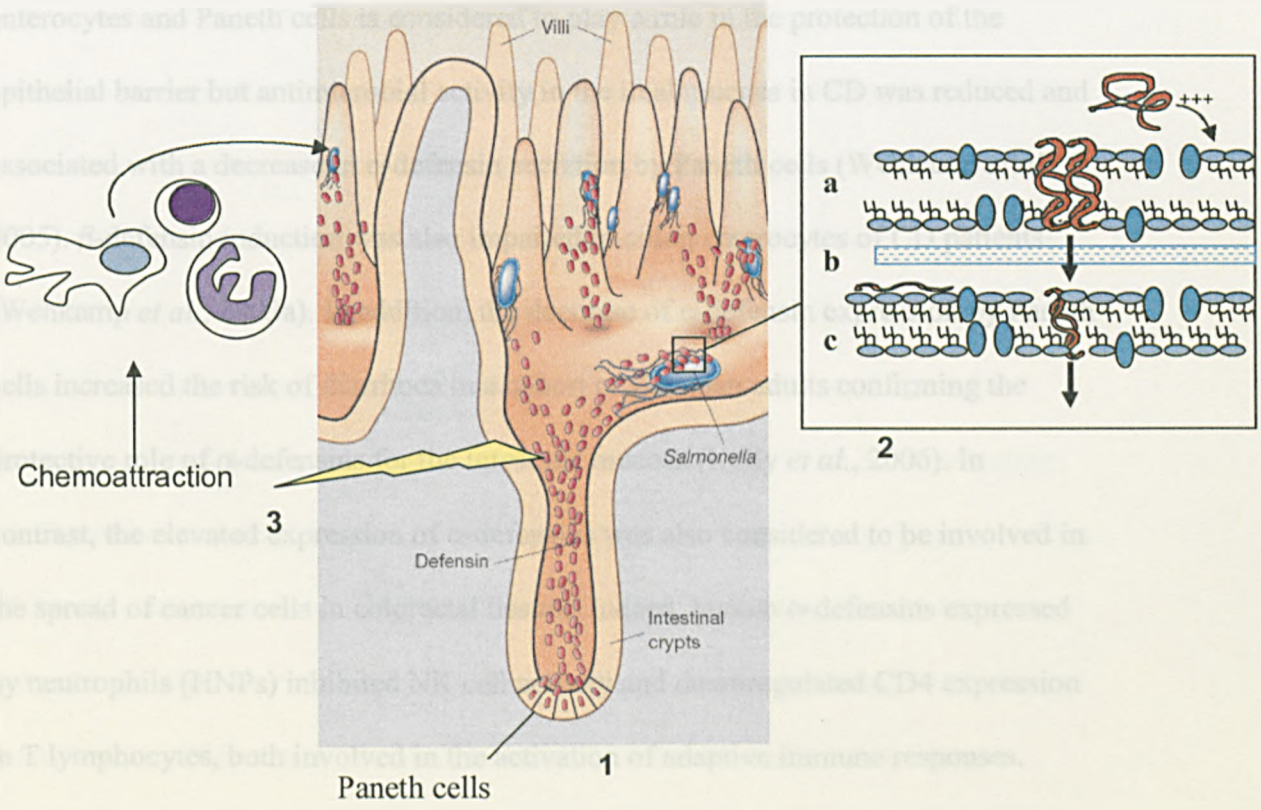
Most defensins exhibit antimicrobial activity against bacteria, fungi, parasites and viruses. Under optimal conditions of low ionic strength, and low concentrations of interfering substances such as plasma protein or divalent cations, defensin activity is observed at low concentrations, e.g. 1-10 $\mu g\ ml^{-1}$ (Ganz, 2003a). The avian defensins including gallinacin 1 and 2, were found to be >90% effective against *Escherichia coli*, *S.* Typhimurium and *Listeria monocytogenes* at 16 $\mu g\ ml^{-1}$ (Evans *et al.*, 1995; Sugiarto & Yu, 2004).

The antimicrobial activity of defensins is usually associated with their ability to depolarise and permeabilise microbial membranes (Devine, 2003). In bacteria, defensins appear to bind electrostatically to components of the outer membrane, such as LPS in gram-negative bacteria or teichoic acid in gram-positive bacteria (Wimley *et al.*, 1994). These monomeric peptides, composed of positively charged and

hydrophobic amino acid side chains, aggregate to form multimeric pores in prokaryotic cells and disrupt the bacterial membrane's function (Ganz, 2003a). Therefore, the resistance of bacteria to defensins is dependent on the outer membrane structure and charge density (Devine, 2003). The antimicrobial activities of defensins are essential for the protection of the epithelium against pathogens at the early stage of infection (Wilson *et al.*, 1999). Defensins play an important role in the elimination of intestinal infection since matrylisin-deficient ($MAT^{-/-}$) mice, which lack the ability to produce mature intestinal defensins (cryptidins), showed an increase in susceptibility to S*almonella* infection (Wilson *et al.*, 1999).

However, this is not the only role attributed to defensins. β-defensin 2 (SBD-2) is expressed in the intestine and extra-intestinal tissues such as trachea, kidney and lung of foetal and neonatal sheep, but its expression was only detected in the intestine of adult sheep. Therefore, the wide distribution of expression SBD-2 in young, developing sheep suggested the involvement of β-defensins in cellular growth/differentiation (Meyerholz *et al.*, 2004). In addition, various defensins have chemotactic activity for monocytes, T cells and dendritic cells (Ganz, 2003a). Indeed, in mammals β-defensins are chemotactic through the chemokine receptor CCR6 (Yang *et al.*, 1999) and can act as ligand for Toll-like receptors (TLR). Murine β-defensin 2 acts directly on immature dendritic cells through TLR-4, inducing the release of mediators and dendritic cell maturation (Biragyn *et al.*, 2002). Human neutrophil defensin, HNP-1, also inhibits the activation of the classical complement pathway by binding C1q (van den Berg *et al.*, 1998). This specific signal gives defensins a role in enhancing host immunity by bridging innate and adaptive immunity (Lillard *et al.*, 1999) (Figure 1.4).

Figure 1.4: Biological activities of defensins.
1. Bacteria attach to villi of the intestine and are attacked by defensins secreted from Paneth cells located in the intestinal crypts. (Ganz, 2003b).
2. Interactions between defensins and bacterial cells. Peptides bind to the divalent cation-binding sites on LPS disrupting the outer membrane and allowing passage of defensins through it. Defensins then bind to the interfacial region of the cytoplasmic membrane, aggregate within the membrane causing depolarisation and permeabilisation, allowing some peptides to access to the cytoplasm (adapted from (Devine, 2003). **a**. Outer membrane of bacterial cell; **b.** Peptidoglycan; **c.** Cytoplasmic membrane of the bacterial cell.
3. Defensins are chemotactic for lymphocytes, neutrophils, monocytes and dendritic cells.



Differential expression of defensins also occurs in response to certain diseases. Inflammatory bowel disease, a genetically-dependent chronic inflammation of the intestine, is described as Crohn's disease (CD) or ulcerative colitis (UC) according to the site of inflammation. UC takes place in the colon, while CD occurs at different sites of the intestine. The inflammation is induced by a perturbation in the balance of commensal bacteria in the intestine, which might be triggered by a defect in defensin expression observed in CD patients. Indeed, defensin expression by intestinal enterocytes and Paneth cells is considered to play a role in the protection of the epithelial barrier but antimicrobial activity in the ileal mucosa in CD was reduced and associated with a decrease in α-defensin secretion by Paneth cells (Wehkamp *et al.*, 2005). β-defensin induction was also impaired in colon enterocytes of CD patients (Wehkamp *et al.*, 2003a). In addition, the decrease of α-defensin expression by Paneth cells increased the risk of diarrhoea in a cohort of Zambian adults confirming the protective role of α-defensins for the intestinal mucosa (Kelly *et al.*, 2006). In contrast, the elevated expression of α-defensins was also considered to be involved in the spread of cancer cells in colorectal tissues. Indeed, human α-defensins expressed by neutrophils (HNPs) inhibited NK cell activity and downregulated CD4 expression in T lymphocytes, both involved in the activation of adaptive immune responses. Therefore, the elevated concentration of HNPs observed in human colorectal cancer tissues suggests that HNPs might downregulate the adaptive immune response and favour evasion of that response by cancer cells (Zhang *et al.*, 2004).

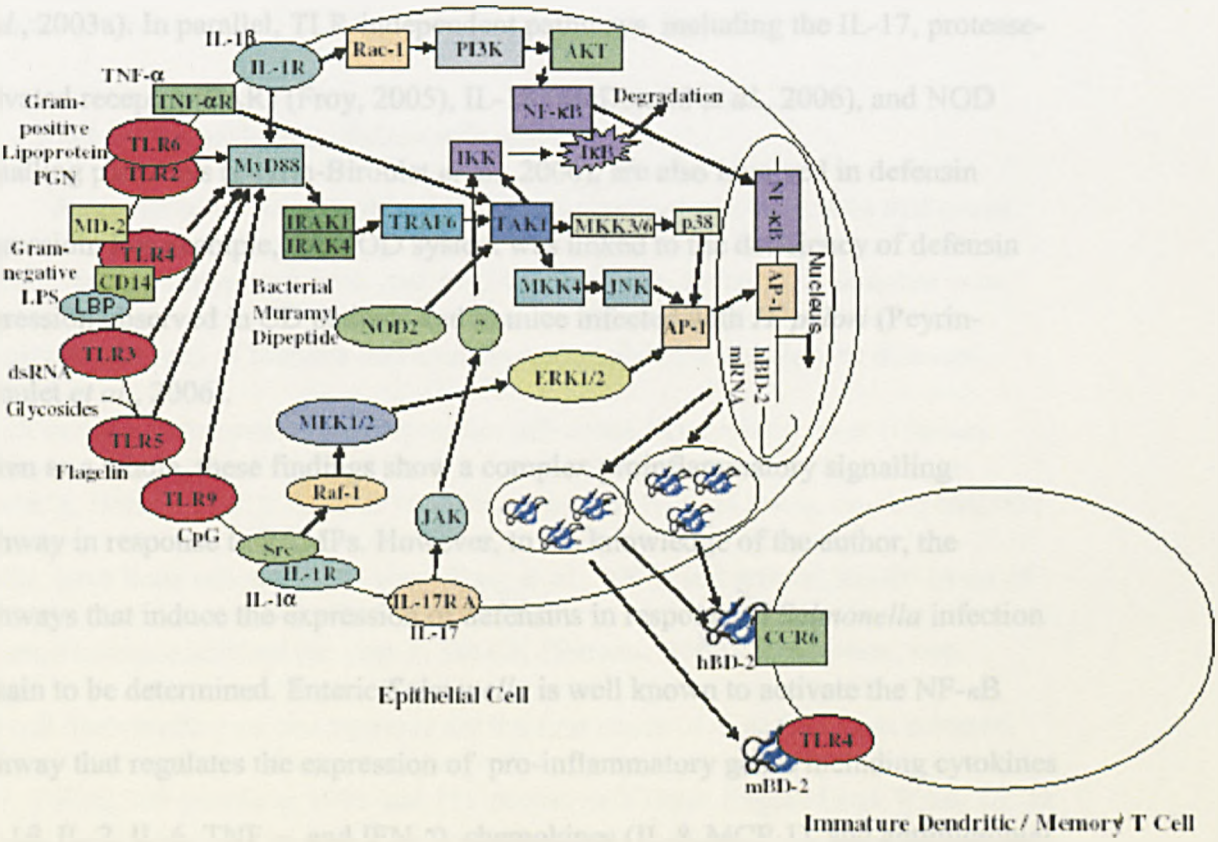
1.2.4 Regulation of defensin expression

A variety of signalling pathways inducing the expression of innate effector molecules, such as defensins, in response to pathogen infection has been identified

(Figure 1.5). They can be classified as TLR-dependent and independent pathways (Peyrin-Biroulet *et al.*, 2006).

In the lung, stimulation with peptidoglycan or lipopeptide increases TLR2 expression and induces hBD-2 expression, which reduces bacterial counts (Hertz *et al.*, 2003; Homma *et al.*, 2004). Other TLRs are involved in the increase of HBD-2 expression in response to different ligands, such as TLR3 in response to synthetic dsRNA (Duits *et al.*, 2003), TLR4 stimulated by LPS (Jia *et al.*, 2004) and TLR9 after stimulation with microbial DNA (Platz *et al.*, 2004). Other tissues have also been shown to express defensins after stimulation of certain TLRs. Intestinal cells express hBD-2 expression after stimulation of TLR2 and TLR6 with peptidoglycan (Vora *et al.*, 2004) and TLR5 with *Salmonella enteritidis* flagellin (FliC) (Ogushi *et al.*, 2001) and uterine epithelial cells express hBD-2 and hBD-1 in response to the TLR3 agonist poly(I:C) (Schaefer *et al.*, 2004). The TLR-dependent pathway also induces the expression of α-defensins. Human natural killer cells express TLRs on their surface, which were stimulated with TLR2 and TLR5 ligands, outer membrane protein A from *Klebsiella pneumoniae* and flagellin respectively, inducing the secretion of α-defensins (Chalifour *et al.*, 2004). All these studies show the importance of TLRs in the induction of defensin expression. In addition, downstream TLR-signalling pathways, including the NF-κB signalling pathway, the IL-1R signalling pathway and the MAPK signalling pathway, are involved in defensin expression. Stimulation of TLR triggers a signalling cascade leading to increased expression of proinflammatory genes via transcriptional factors such as NF-κB, activated by the NF-κB pathway, and AP-1, activated by the MAPK pathway. By contrast, the IL-1R, which shares homology with TLRs, activates both NF-κB and MAPK signalling pathways. In epithelial cells, IL-1β up-regulates hBD-2 via activation of the transcription factor

Figure 1.5: Regulation of HBD-2 expression via TLR-dependent and independent signalling pathway (Froy, 2005). TLRs (red) induce HBD2 expression by activating NF-κB via the MyD88-dependent pathway. IL-1R (blue) activates NF-κB (purple) via the MyD88-dependent pathway, the P13K pathway and the ERK pathway. IL-17R (yellow) activates NF-κB (purple) via the JAK pathway. NOD2 (green) induces HBD2 by mediating NF-κB (purple), MAPK/p38 and MAPK/ERK activation via TAK1. HBD2 can also act as a ligand for CCR6 (green) to chemoattract DC and memory T cells or TLR4 (red) to activate DCs.



NF-κB as well as activation of signalling proteins, particularly MAPK/PKC, p38 MAPK, MAPK/JNK and PI3K (Jang *et al.*, 2004). The MAPK/ERK pathway is also involved in hBD-2 and -3 expression and the MAPK/JNK pathway plays a crucial role in defensin expression in response to *Helicobacter pylori* (Boughan *et al.*, 2006). Similarly, the MAPK/ERK and p38 MAPK pathways increase hBD-2 expression in response to *S.* Enteritidis flagellin (FliC) (Ogushi *et al.*, 2004). Finally, the p38 MAPK pathway was also involved in the production of α-defensins in mice (Salzman *et al.*, 2003a). In parallel, TLR-independent pathways, including the IL-17, protease-activated receptor (PAR) (Froy, 2005), IL-18 (McDonald *et al.*, 2006), and NOD signalling pathways (Peyrin-Biroulet *et al.*, 2006), are also involved in defensin expression. For example, the NOD system was linked to the deficiency of defensin expression observed in CD patients and in mice infected with *H. pylori* (Peyrin-Biroulet *et al.*, 2006).

Taken as a whole, these findings show a complex proinflammatory signalling pathway in response to PAMPs. However, to the knowledge of the author, the pathways that induce the expression of defensins in response to *Salmonella* infection remain to be determined. Enteric *Salmonella* is well known to activate the NF-κB pathway that regulates the expression of pro-inflammatory genes including cytokines (IL-1β, IL-2, IL-6, TNF-α, and IFN-γ), chemokines (IL-8, MCP-1), and antimicrobial peptides involved in the inflammatory response such as the recruitment of PMNs (Sun *et al.* 2005). These data suggest that defensins may also be induced via NF-κB pathway. Similarly, the MAPK/ERK and p38 MAPK pathways may be involved in the regulation of transcription of defensins in response to *Salmonella* infection *in vivo* as they were described to be involved in the increase of hBD-2 expression in response to *S.* Enteritidis flagellin (FliC) *in vitro* (Ogushi *et al.*, 2004). By contrast, the

inhibition of α-defensins expressed by Paneth cells has been recently described involving the activation of the p38 MAPK pathway in response to *Salmonella* infection (Salzman *et al.* 2003a). These suggest that α- and β-defensins may be regulated by different pathways, which might be activated in TLRs-dependent manner. Therefore, further investigation of inducible defensins other than hBD-2, which has been extensively studied, will allow understanding of the synergy and/or redundancy between the TLR-dependent and -independent pathways in regulation of defensin production.

1.3 A food-borne pathogen, *Salmonella enterica*

Salmonella are rod-shaped motile Gram-negative enterobacteria that cause typhoid fever, paratyphoid fever and food-borne illness. *Salmonella enterica* is an important pathogen of humans and animals responsible for a variety of diseases, which include gastroenteritis and systemic infections like typhoid fever (Hansen-Wester & Hensel, 2001). Around 16 millions case of typhoid fever, causing 600,000 deaths, have been estimated per year (Pang *et al.*, 1995) and around 30,000 cases of salmonellosis are notified per year in the UK (Barrow, 2000). In addition, non-typhoid *Salmonella enterica* serovars are the first cause of mortality from infected food, killing 268 people in 1995 and 119 people in 2000 in England and Wales (Adak *et al.*, 2002). Classification of *Salmonella* spp. results in around 2100 serovars, named according to the host and the disease symptoms or the place they were originally found. However, there is high percentage identity at the genomic level between many serovars, leading to the proposition that most *Salmonella* serovars should be grouped under *Salmonella enterica* species (Edwards *et al.*, 2002). *Salmonella enterica* can be then classified in two groups according to the nature of the infected host. The broad-host range *Salmonella*, such as *S.* Typhimurium and *S.* Enteritidis, infect human, mice

and chickens and cause different infections according to the host, such as gastroenteritis in humans, systemic infection in mice and asymptomatic infection in certain chicken lines. The second group, or "host-specific" serovars, generally infects only a single host, such as *S.* Dublin infecting cattle, *S.* Typhi infecting human and *S.* Gallinarum or *S.* Pullorum infecting chickens, while they rarely cause disease in other animals (Edwards *et al.*, 2002). Consequently, *Salmonella* have also been used as a model to study host-pathogen interactions at the molecular level, particularly *S.* Typhimurium that causes gastroenteritis in human and typhoid-like disease in mice (Hansen-Wester & Hensel, 2001).

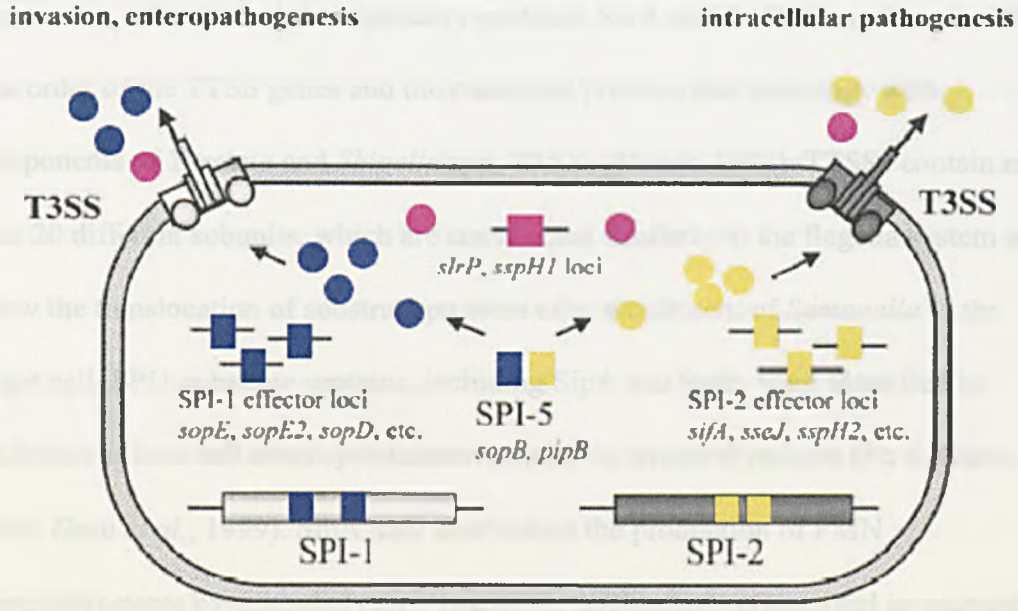
1.3.1 Elements of *Salmonella enterica* virulence

Salmonella have a complex life cycle in infected animals with two main virulence characteristics, including invasion of epithelial cells and survival and replication in phagocytic cells. In addition, food poisoning serovars are able to colonise the alimentary tract of food animals in the absence of clinical disease. This characteristic is poorly understood, although genes contributing to colonisation are being identified (Morgan *et al.*, 2004; Turner *et al.*, 1998). The extent to which interaction with the host is a component of this is controversial. The penetration of intestinal epithelial cells is an initial step for *Salmonella* after an oral infection. For this purpose, the bacteria adhere to and invade the epithelial cells using different mechanisms encoded by a large number of genes implicated in *Salmonella* virulence. The survival and replication of *Salmonella* inside phagocytic cells, particularly inside the phagosomal vacuole, also requires a complex mechanism to resist environmental changes, such as decrease in pH, nutritional deprivation, oxidative burst and secretion of AMPs (Hansen-Wester & Hensel, 2001). *Salmonella* spp. genome analysis reveals

many insertions, named islands, which were presumably acquired via horizontal gene transfer (Edwards *et al.*, 2002). Several virulence determinants are are encoded by these islands, which are called *Salmonella* pathogenicity islands (SPI). SPIs are large segments of DNA only present in pathogenic species, which can transform a nonpathogenic species into a pathogen after insertion in their genome. To date, seventeen SPIs have been identified in *Salmonella* (Vernikos & Parkhill, 2006), including SPI1 involved in *Salmonella* invasion (Galan & Curtiss, 1989), SPI2 in the survival and replication of *Salmonella* inside phagocytic cells (Ochman *et al.*, 1996), SPI3, containing the high-affinity magnesium transport system MgtCB, which is important for intracellular replication of *Salmonella* in the nutrient-poor environment of the phagosome (Blanc-Potard *et al.*, 1999), SPI4, including a macrophage survival locus (Wong *et al.*, 1998) and SPI5, harboring effector proteins of SPI1 and SPI2 (Knodler *et al.*, 2002) and involved in *Salmonella* enteropathogenesis (Blanc-Potard *et al.*, 1999). Five other SPI were then identified in the *S. enterica* serovars Typhi and Typhimurium, named SPI6 to SPI10 (McClelland *et al.*, 2001; Parkhill *et al.*, 2001). SPI11 and SPI12 were identified in *S.* Choleraesuis (Chiu *et al.*, 2005) and SPI13 and SPI14 in *S.* Gallinarum (Shah *et al.*, 2005). More recently, three other SPIs were identified, SPI15, SPI16 and SPI17. Interestingly, SPI17 is absent from the genome of *S.* Typhimurium LT2, SPI16 is present in all the *Salmonella* lineages tested except *Salmonella enterica* subsp. *arizonae, Salmonella bongori* and *E. coli*, and SPI15 is only present in *S.* Typhi CT18 and TY2, and *Shigella flexneri* (Vernikos & Parkhill, 2006).

However, only SPI1 and SPI2 encode functionally related genes, including the type III secretion systems (TTSSs) (Figure 1.6). SPI1 is 40 kb in length and encodes 39 genes including those encoding Mxi and Spa proteins involved in *Shigella* invasion of

Figure 1.6: Relationship between SPI1, SPI2 and SPI5 functions of *S. enterica*. Effector proteins (○) from SPI1 system (blue □) are translocated by the SPI1 TTSS and Effector proteins (○) from SPI2 system (yellow □) are translocated by the SPI2 TTSS. SPI-5 contains genes encoding effector proteins for the SPI1 and the SPI2 system. Substrate proteins (pink ○) are secreted by the SPI1 and SPI2 TTSS (Schmidt & Hensel, 2004).



eukaryotic cells, proteins involved in TTSS function, including proteins required for the needle complex and secreted effectors (Hansen-Wester & Hensel, 2001), and regulatory proteins, such as HilA, HilC, HilD and InvF, which are induced according to the host environment (Ellermeier & Slauch, 2007). SPI2 is also 40 kb in size and contains 4 operons encoding proteins involved in TTSS function, molecular chaperones, effector and the regulatory proteins, SsrA and SsrB (Hensel *et al.*, 1998). The order of the TTSS genes and their secreted proteins has homology with components of *Yersinia* and *Shigella* spp. TTSSs (Hueck, 1998). TTSSs contain more than 20 different subunits, which are constructed similarly to the flagella system and allow the translocation of substrate proteins after attachment of *Samonella* to the target cell. SPI1 substrate proteins, including SipA and SptP, were identified as inhibitors of host cell actin cytoskeleton to lead the invasion process (Fu & Galan, 1999; Zhou *et al.*, 1999). SipA may also induce the production of PMN chemoattractants by epithelial cells (Lee *et al.*, 2000), SipB is involved in macrophage apoptosis and the subset of SopA, SopB and SopD are involved in the secretion of chloride and loss of fluid into the intestinal lumen contributing to the diarrheal phenotype in calves (Zhang *et al.*, 2002). Although the contribution of most of the SPI2-translocated proteins has yet to be established (Waterman & Holden, 2003), SpiC inhibits cellular trafficking (Uchiya *et al.*, 1999), the SifA protein is required for *Salmonella*-containing vacuole (SCVs) membrane integrity and regulated by the two-component regulatory system SsrAB (Beuzon *et al.*, 2000), and SseF and SseG contribute to the formation of *Salmonella*-induced filaments (Sif) (Kuhle & Hensel, 2002) and are also necessary for perinuclear localization of SCVs (Deiwick *et al.*, 2006). Interestingly, phylogenetic analysis showed that SPI-1 sequences were identified in all *Salmonella* species, despite secondary loss in certain *S. enterica*. In

contrast, SPI2-like sequences were not identified in *S. bongori,* which is the most divergent lineage of *Salmonella* (Groisman & Ochman, 1996), suggesting that SPI2 might have integrated into the *Salmonella* genome later, as a crucial event in *Salmonella*'s evolution as an intracellular pathogen.

More recently, several genes involved in enteritis and systemic disease were analysed using a modified differential fluorescence induction technique (Rollenhagen & Bumann, 2006). In enteritis disease models, specific genes involved in the activation of SPI-1, flagellum synthesis, anaerobic growth of *Salmonella* and in the utilisation of ferrichrome siderophores due to the limited avaibility of iron during gut invasion were identified. In comparison, in a systemic disease model, genes involved in the activation of SPI2, in the regulation of PhoPQ system that induces changes of *Salmonella* membrane surface to be resistant to antimicrobial peptides and in the production of different nucleases to modulate mRNA turnover for regulation of virulence gene expression, were analysed. Therefore, this gene-disease specific analysis suggests that these specific genes may be involved in *Salmonella in vivo* infection.

1.3.2 Host-*Salmonella* interactions

In mammals, *Salmonella* invasion of host epithelial cells requires SPI1 effector proteins to change the host cell cytoskeleton. After internalization of the bacteria, the SPI2 TTSS allows the replication of *Salmonella* in the lamina propria. The invasion process induces a proinflammatory response characterised by the release of cytokines, chemokines and antimicrobial peptides, either by stimulating TLR-independent pathways at the entrance of the bacteria into epithelial cells or by activating TLR signalling pathways in the lamina propria. Indeed, an *S.* Typhimurium

SPI2 mutant (but with functional SPI1) caused colitis in MyD88$^{-/-}$ mice, excluding the stimulation of MyD88-dependent TLR signalling pathways. In parallel, an *S.* Typhimurium SPI1 mutant (but with functional SPI2) did not cause colitis in MyD88$^{-/-}$ mice, despite its presence in the lamina propria, suggesting the stimulation of MyD88-dependent TLR signalling pathways by *S.* Typhimurium during its replication in the lamina propria (Hapfelmeier *et al.*, 2005). In addition, the absence of a proinflammatory stimulus by commensal bacteria, particularly in the intestine, could be explained by the lack of TLR4 and TLR2 expression on the surface of intestinal epithelial cells, the expression of TLR5 only on the basolateral surface of epithelial cells and by the secretion of antimicrobial peptides by epithelial cells creating a protective barrier (Mahida & Rolfe, 2004).

Different roles, including the regulation of commensal bacteria and the protection of the epithelial crypt from enteric pathogens, have been attributed to AMPs. If the first role is difficult to evaluate, the protective role against pathogen, particularly *Salmonella*, has been demonstrated. Indeed, mice deficient in matrilysin 7, MMP-7, involved in cryptidin processing, were more susceptible to *Salmonella* infection than wild-type mice (Wilson *et al.*, 1999). In addition, macrophages are a reservoir of *Salmonella* Typhimurium infections causing systemic disease in mice. A recent study showed that inhibition of *S.* Typhimurium cell division was the result of antimicrobial effector expression, such as cathelicidin induced by an increase of intracellular reactive oxygen intermediates (Rosenberger *et al.*, 2004). Finally, transgenic mice expressing human defensin 5 (HD-5), an α-defensin secreted by human Paneth cells, were infected orally with *S.* Typhimurium. Interestingly, HD-5 transgenic mice showed few signs of illness and recovered after 12 h, while the wild-type control mice reached a mortality rate of 100% by 24 h, suggesting that the expression of α-

defensins by Paneth cells conferred resistance to *Salmonella* infection by increasing innate immune efficiency (Salzman *et al.*, 2003b).

However, a few studies have shown that *Salmonella* can be resistant to cationic antimicrobial peptides (CAMP), which can be classified as α-helical peptides, such as C18G peptide, and β-sheet peptides such as the defensins and protegrins. Indeed, the presence of sublethal doses of CAMP, including the α-helical peptide C18G and the β-sheet peptide protegrin-1, induces PhoP (Bader *et al.*, 2003), which is one component of the PhoPQ regulator system able to translate environmental signals into changes in gene expression. Activation of the PhoPQ system induced the repression of the SPI1 TTSS genes, inhibiting the invasion of *S.* Typhimurium (Rakeman & Miller, 1999) and changing the bacterial cell surface. Modification of *S. enterica* LPS was observed at the anionic lipid A level. The *pmrE* and *pmrHFIJKL* genes are involved in the production of aminoarabinose, which is then inserted into lipid A. This modification of the LPS core alters the net charge of the bacterial surface and reduces antimicrobial peptide binding. The incorporation of fatty acid into lipid A, which is dependent on the *pagP* gene, reduces the permeability of the *S. enterica* outer membrane to CAMP. In addition to the bacterial cell surface changes, *S. enterica* produces a protease, PgtE, that cleaves the α-helical CAMP (Peschel, 2002), and a Mig-14 protein associated with the inner membrane of *S. enterica*, which is essential in CAMP resistance (Brodsky *et al.*, 2005). Therefore, CAMP might be environmental signals to stop the invasion of epithelial cells and induce a complex response, allowing *S.* Typhimurium to resist innate immune mechanisms (Bader *et al.*, 2003). Salzmann *et al.* showed that the inhibition of α-defensin expression by Paneth cells by *S.* Typhimurium was SPI1 TTSS dependent, via the activation of the p38 MAPK pathway (Salzman *et al.*, 2003a). Therefore, resistance to CAMP, by altering

the LPS surface or by inhibiting CAMP expression, involves the secretion of effector molecules by *Salmonella* to modulate the host innate immune response via cellular signalling pathways. The virulence mechanisms involved need further studies but, so far, the SPI1 TTSS seems to be essential.

In the chicken, the molecular mechanisms involved in *Salmonella* invasion are little known. The *S.* Enteritidis *yfg-eng* locus, composed of four open reading frames including *yfgM*, *yfgL*, *engA* and *yfgJ*, is involved in chicken colonisation, particularly *yfgL*. Indeed, invasion of a *yfgL* mutant was reduced *in vivo* and in avian HD11 macrophage-like cells. The motility and the secretion of SPI1 and flagellar proteins was also abnormal, suggesting a role for the *S.* Enteritidis *yfg-eng* locus in the regulation of SPI1 TTSS and flagellar TTSS (Amy *et al.*, 2004). In addition, the importance of the YfgL outer membrane lipoprotein was also observed *in vitro* and *in vivo* in mice, with the same characteristics as shown in chickens (Fardini *et al.*, 2007). Recently, virulence genes of *S.* Gallinarum were identified *in vivo* (Shah *et al.*, 2005). SPI1, SPI2 and SPI10 mutants were analysed, as well as mutants in the newly identified SPI13 and SPI14. SPI1 and SPI2 contributed to *S.* Gallinarum virulence in chickens. Indeed, SPI1 seems to be important at the early stage of intestinal invasion and to initiate systemic infection in young chickens. Interestingly, the SPI2 genes involved in *S.* Gallinarum virulence in chickens are different from the SPI2 genes involved in *S.* Typhimurium chicken colonisation, suggesting different mechanisms of virulence. SPI10, which codes for fimbriae proteins involved in the adhesion of *Salmonella* to host cells, also plays a role in *S.* Gallinarum virulence. Finally, SPI13 and SPI14 have not yet been shown to be involved in *S.* Typhimurium virulence and not all of the genes they encode have been characterised. Except for the SPI13 *gacD* gene, that shares 100% homology with the *cat-2* gene involved in invasion and

survival of *S. enterica* in chicken macrophages, the role of the other genes in *S. enterica* virulence is still unknown. However, the specific discovery of these new SPIs in *S.* Gallinarum might suggest they have an important role in the virulence of this non-motile, avian host-adapted serotype, compared to all motile *Salmonella* serotypes (Shah *et al.*, 2005).

However, the mechanism by which host-specific *S.* Gallinarum and *S.* Pullorum cause systemic disease and death, compared to broad-host range *Salmonella* such as *S.* Typhimurium, which cause asymptomatic infection, is still unknown (Chadfield *et al.*, 2003). The intestinal invasion of *Salmonella* serotypes is highest in the caecal tonsil, composed of lymphoid cells, but the quantitative measure used did not detect any difference in intestinal colonisation between host-specific and broad-range *Salmonella* (Chadfield *et al.*, 2003). In addition, *S.* Gallinarum did not survive in greater numbers in HD11 macrophage-like cells compared to other *Salmonella* serotypes. However, survival in macrophages should be essential for host-specific serotypes to cause systemic disease in chickens.

Previous studies identified certain chicken lines as resistant to systemic salmonellosis, such as lines Wl, 6_1 and N, while other lines were susceptible, including lines C, 7_2 and 15I (Bumstead & Barrow, 1988; Bumstead & Barrow, 1993). Interestingly, macrophages from the resistant lines were more efficient at clearing *S.* Gallinarum, compared to macrophages from the susceptible lines (Wigley *et al.*, 2002). The resistance/susceptibility pattern is genetically dependent, at least partially encoded by the *SAL1* locus mapped to chromosome 5 (Mariani *et al.*, 2001), and resistant-line macrophages showed a higher oxygen-dependent antimicrobial activity (Wigley *et al.*, 2002). In contrast, differences in *S.* Typhimurium colonisation of the intestine is not related to the *SAL1* locus. Line 6_1 chickens, resistant to *Salmonella* colonisation,

showed higher numbers of circulating heterophils compared to line N chickens (Barrow *et al.*, 2004). As discussed earlier, heterophils secrete AMPs that may play a role in resistance to gut colonisation (Wigley, 2004). Cytokines and chemokines have been already analysed in the gastrointestinal gut (Withanage *et al.*,2005; Beal *et al.*, 2004; Haghighi *et al.*, 2007) and showed a correlation between their expression profiles and the resistance to *S.* Typhimurium in the chicken (Table 1.4). In addition, chicken TLRs are involved in resistance to *Salmonella* infection as TLRs are in mammals. TLR4 is involved in the resistance/susceptibility of chickens to *Salmonella* infection (Leveque *et al.*, 2003) and TLR5 restricts the entry of flagellated *Salmonella* into the systemic site of chickens (Kogut *et al.*, 2005). Finally, *Salmonella* infection upregulates TLR15 in the chicken caecum, as previously observed for TLR2 (Higgs *et al.*, 2006). TLR are an important component in the regulation of defensin expression and the TLR signalling pathway tends to be targeted by *Salmonella* to resist AMP in mammals. Therefore it will be interesting to analyse the expression of chicken defensins or gallinacins, as this ancient component of innate immunity seems to play a crucial role in *Salmonella* invasion in mammals, and its role might be similar in chickens.

1.4 Project aims

Salmonella enterica is a facultative intracellular pathogen, usually motile and the primary cause of food poisoning, mainly through consumption of infected poultry meat and eggs, in Europe. Salmonellosis in chickens can be induced either by broad host-range *Salmonella* such as *S.* Typhimurium or host-specific serotypes such as *S.* Gallinarum. Mammalian defensins play an important role in innate immune defence, particularly in response to gastrointestinal infection such as salmonellosis. Gallinacins

Table 1.4: Cytokines/ chemokines expression in response to *Salmonella* infection in the gastrointestinal tract of chickens. Y, Expression up-regulated; N, No significantly change in expression observed; ND, not detected in the experiment.

Cytokines/ chemokines		References
IL-1β	N	Whitanage, 2005; Beals, 2004
IL-4	ND	Whitanage, 2005
IL-6	N	Whitanage, 2005; Haghighi, 2007
IL-10	N	Whitanage, 2005
IL-12	Y (at 1 and 5 dpi)	Haghighi, 2007
IFN-γ	Y (depending of the chicken age at the time of infection)	Whitanage, 2005; Beals, 2004; Haghighi, 2007
TGF-β4	Y (at 1 dpi)	Whitanage, 2005; Beals, 2004
Chemokine K60	N	Whitanage, 2005
IL-8	ND	Whitanage, 2005
MIP-1β	N	Whitanage, 2005

are the chicken's β-defensins, expressed by heterophils (the avian neutrophil equivalent) and epithelial cells, and therefore presumably play a role in the chicken's innate immune response against *Salmonella* infection. The project aim was to compare AvBDs mRNA levels in different chicken lines considered as susceptible and resistant to different *Salmonella* serotypes. Indeed, a previous study showed that α-defensins expressed in Paneth cells were inhibited after *Salmonella* infection in mice suggesting that the inhibition defensins is a virulence strategy of the intestine pathogen. Therefore, we wished to test the hypothesis that the susceptibility of chicken lines to *Salmonella* infection correlated with decreased AvBD transcript levels, as previously shown in mice.

In the present study, inbred chicken lines 6_1 and N, previously characterized for their resistance to systemic disease and their differences in levels of *Salmonella* colonisation, were selected to analyse gallinacin expression. Line 6_1 and line N chickens are resistant and susceptible to *Salmonella* serovar Typhimurium colonisation respectively (Barrow *et al.*, 2004; Beal *et al.*, 2005). An important difference in response between the lines

was the number and activity of circulating heterophils, suggesting the involvement of these cells and their secreted components, such as avian β-defensins, in resistance to gut colonization. Therefore, gallinacins 1/1α and 2, originally isolated from heterophils, were chosen to study their expression in chickens. However, a role for β-defensins expressed by epithelial tissues cannot be ruled out and gallinacin 3 was therefore also studied. In addition, three other gallinacins were selected. Identified originally by Dr N. Bumstead at IAH, two were then described as gallinacins 7 and 9 (Lynn *et al.*, 2004), while a third new defensin, not yet published, was characterised in this study and named gallinacin 14.

Recently, a new nomenclature for chicken β-defensins has been proposed, using the numbering system of Xiao *et al.* (2004) and changing the term "gallinacin" into "avian beta-defensin", abbreviated AvBD (Lynn *et al.*, 2007) (Table 1.4). This nomenclature will be used from now on throughout this thesis.

In this study, AvBD genomic DNAs were first characterised and cloned in an appropriate vector to determine the structure of the genes and to enable the design of primers and probes to study AvBD expression at the mRNA level using real-time RT-PCR. The potential development of bioreagents, such as monoclonal antibodies, to visualize AvBD expression by histochemistry in tissues of uninfected chickens and chickens infected with *Salmonella*, required the production of AvBD peptides, which would also permit testing of the killing and chemotactic activities of the AvBDs. The second part of the project was then to clone AvBDs 1/1α, 2, 3, 4, 5 and 14 into an expression vector pTriEx1.1, and to express AvBD peptides using the baculovirus expression system designed by Prof I. Jones at the University of Reading. However, the specific physicochemical characteristics of these antimicrobial peptides makes obtaining biologically active AvBD peptides difficult. Therefore, the expression of AvBDs at the mRNA level was analysed. A panel of tissues including lung, bursa, bone marrow, jejunum, ileum, ceaca wall, ceacal tonsil and skin was collected from uninfected chickens to assess the distribution of AvBD expression. In addition, two lines of chickens, line 6_1 and N, were infected with *S.* Typhimurium to analyse AvBD 1/1α, 2, 3, 4 and 5 expression in chickens both resistant and susceptible to *Salmonella* colonisation. In parallel, production of AvBDs in resistant and susceptible chickens following systemic infection was analysed to assess their expression in response to host-specific *Salmonella* and broad-host range *Salmonella* serotypes.

Table 1.5: New nomenclature of chicken β-defensins (Lynn *et al.*, 2007).

New gene/protein name	Lynn/Higgs *et al.* definition	Xiao *et al.* definition	RefSeq definition	Accession no.
Avian beta-defensin 1 (AvBD1)	Gallinacin 1 (GAL1)	Gallinacin 1 (GAL1)	Gallinacin 1 (GAL1)	NM 204993
Avian beta-defensin 2 (AvBD2))	Gallinacin 2 (GAL2)	Gallinacin 2 (GAL2)	Gallinacin 2 (GAL2)	NM 204992
Avian beta-defensin 3 (AvBD3)	Gallinacin 3 (GAL3)	Gallinacin 3 (GAL3)	Beta-defensin prepropeptide (GAL3)	NM 204650
Avian beta-defensin 4 (AvBD4)	Gallinacin 7 prepropeptide(GAL7)	Beta-defensin 4 (GAL4)	GAL 4 (GAL4)	NM 001001610
Avian beta-defensin 5 (AvBD5)	Gallinacin 9 prepropeptide(GAL9)	Beta-defensin 5 (GAL5)	GAL 5 (GAL5)	NM 001001608
Avian beta-defensin 6 (AvBD6)	Gallinacin 4 prepropeptide(GAL4)	Beta-defensin 6 (GAL6)	GAL 6 (GAL6)	NM 001001193
Avian beta-defensin 7 (AvBD7)	Gallinacin 5 prepropeptide(GAL5)	Beta-defensin 7 (GAL7)	GAL 7 (GAL7)	NM 001001194
Avian beta-defensin 8 (AvBD8)	Gallinacin 12 prepropeptide(GAL12)	Beta-defensin 8 (GAL8)	GAL 8 (GAL8)	NM 001001781
Avian beta-defensin 9 (AvBD9)	Gallinacin 6 prepropeptide(GAL6)	Beta-defensin 9 (GAL9)	GAL 9 (GAL9)	NM 001001611
Avian beta-defensin 10(AvBD10)	Gallinacin 8 prepropeptide(GAL8)	Beta-defensin 10 (GAL10)	GAL 10 (GAL10)	NM 001001609
Avian beta-defensin 11(AvBD11)		Beta-defensin 11 (GAL11)	Gallinacin 11 (GAL11)	NM 001001779
Avian beta-defensin 12(AvBD12)	Gallinacin 10 prepropeptide(GAL10)	Beta-defensin 12 (GAL12)	Beta-defensin 12 (GAL12)	NM 001001607
Avian beta-defensin 13(AvBD13)	Gallinacin 11 prepropeptide(GAL11)	Beta-defensin 13 (GAL13)	Beta-defensin 13 (GAL13)	NM 001001780
Avian beta-defensin 14(AvBD14)			Gallinacin 14 (Gal14)	AM402954

Chapter 2 - Materials and Methods

2.1 Bacterial strains, plasmids, restriction enzymes and oligonucleotides

The bacterial strains used in this thesis are listed in Table 2.1. All were grown aerobically in SOC medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) or in Luria-Bertani (LB) broth or agar for 24 h at 37°C. Where appropriate, ampicillin was added to the medium at 50 μg/ml. For long-term storage, 150 μl of glycerol was added to 850 μl of overnight culture and stored at -80°C.

Table 2.1: *E. coli* strain characteristics.

Strain	Genotype	Antibiotic resistance	Source
TOP10F'	F′ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	None	Invitrogen
DH5α	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	None	Invitrogen
BUE55	Unavailable- originally isolated because of its sensitivity to polymoxin B.	None	Dr D. Devine, University of Leeds

The plasmids used are listed in Table 2.2 and were stored at -20°C.

Table 2.2: Characteristics of plasmids used for cloning.

Plasmids	Description	Antibiotic resistance	Source
pCR 2.1-TOPO	3.9 kb cloning vector (see Appendix 1)	ampicillin and kanamycin	Invitrogen
pTriEx-1.1	5.3 kb expression vector, which can be used in multiple expression systems: *E .coli*, insects and vertebrate cells. It carries optional C-terminal His•Tag and HSV•Tag sequences (see Appendix 2).	ampicillin	Novagen

The restriction enzymes used are listed in Table 2.3 and they were stored at -20°C.

Table 2.3: Characteristics of restriction enzymes (RE) used for cloning.

RE	Description	Buffer
*Nco*I	5′-C^C A T G G-3′ 3′-G G T A C^C-5′	1x NEBuffer 4 (20 mM Tris-acetate; 50 mM potassium acetate; 10 mM Magnesium Acetate;1 mM Dithiothreitol; pH 7.9)
*Bsa*I	5′-G G T C T C N^N N N N-3′ 3′-C C A G A G N N N N N^-5′	1x NEBuffer 3 (50 mM Tris-HCl; 100 mM NaCl; 10 mM $MgCl_2$; 1 mM Dithiothreitol; pH 7.9)
*Bgl*II	5′-A^G A T C T-3′ 3′-T C T A G^A-5′	1x NEBuffer 3 (50 mM Tris-HCl; 100 mM NaCl; 10 mM $MgCl_2$; 1 mM Dithiothreitol; pH 7.9)
*Xho*I	5′-C^T C G A G-3′ 3′-G A G C T^C-5′	1x NEBuffer 2 (10 mM Tris-HCl; 50 mM NaCl; 10 mM $MgCl_2$; 1 mM Dithiothreitol; pH 7.9)
*Nco*I/*Bgl*II		1x NEBuffer 3
*Nco*I/*Xho*I		1x NEBuffer 2
*Bsa*I/*Bgl*II		1x NEBuffer 3
*Bsa*I/*Xho*I		1x NEBuffer 3

The oligonucleotides used to obtain the genomic sequences of AvBDs 1, 2 and 3, the cDNAs of the AvBD and for sequencing reactions are listed in Table 2.4. In order to obtain the genomic DNA of AvBDs 1, 2 and 3, the oligonucleotides were designed according to the cDNA sequences already published. AvBD 1 and 1α differ by only 3 amino acids. The primers are therefore similar for both defensins. The forward primers used to clone the AvBD cDNAs into the expression vector pTriEx 1.1 were designed with a *Bsa*I or *Nco*I restriction site at the 5′ end of the sequence. A *Bgl*II restriction site was also introduced into the 5′end of the reverse primers for AvBDs 3 and 5.

Table 2.4: Oligonucleotide sequences used in cloning and sequencing AvBDs. Underlined nucleotides are the restriction sites used to clone AvBD sequences into the pTriEx 1.1 expression vector.

Primers	Forward sequence	Reverse sequence
AvBD1/1α gDNA	5′-ATGCGGATCGTGTACCTGCT-3′	5′-ATCTTGAGGATTTCCCACTGA-3′
AvBD2 gDNA	5′-TCTCTCCTCTTCCTGGCACT-3′	5′-GCCATTTGCAGCAGGAA-3′
AvBD3 gDNA	5′-TCTTGTTTCTCCAGGGTGCT-3′	5′-ATTCAGGGCATCAACCTCATA-3′
AvBD1/1α cDNA	5′-GATCCATGGATGCGGATCGTGTACC TGCT3′	5′-TCAGCCCCATATTCTTTTGC-3′
AvBD1/1α expression	5′-GATGGTCTCACATGCGGATCGT-3′	5′-TCAGCCCCATATTCTTTTGC-3′
AvBD2 cDNA	5′-GAGCCATGGATGAGGATTCTT TACCTGCTTTTC-3′	5′-TATGCATTCCAAGGCCATTT-3′
AvBD2 expression	5′-GATGGTCTCACATGAGGATT-3′	5′-TATGCATTCCAAGGCCATTT-3′
AvBD3 cDNA	5′-GTACCATGGATGCGGATCGTGTAC CTGCTCATCCCCTTCTTCCTCTTG-3′	5′-GATAGATCTTCAATGGGGGTTGTT TCCAGGAGCGAGAAGCCACGGCGA-3′
AvBD3 expression	5′-GATGGTCTCACATGCGGATCGT-3′	5′-GATAGATCTTCAATGGGGGTTGTT TCCAGGAGCGAGAAGCCACGGCGA-3′
AvBD4 cDNA	5′-CTGCCATGAAAATCCTTTGCTT-3′	5′-TTACCATCTACAGCAAGAATA-3′
AvBD4 expression	5′-GATCCATGGCTGCCATGAAAAT-3′	5′-GGAGATCTTTACCATCACAGCAA-3′
AvBD5 cDNA	5′-GATCCATGGATGCAGATCCTGCCTC TCCT-3′	5′-GATAGATCTCTACCTCCGGCAGCA GAAGT-3′
AvBD14 cDNA	5′-ATGGGCATATTCCTCCTGTTTC-3′	5′-TCACCAAAAGGGTCTGCAGCA-3′
AvBD14 expression	5′-GATCCATGGGCATATTCCTCCT-3′	5′-TCACCAAAAGGGTCTGCAGCA-3′
-40 primer (M13)	5′-GTTTTCCCAGTCACGAC-3′	5′-CAGGAAACAGCTATGAC-3′
pTriEx primer	5′-GTTATTGTGCTGTCTCATCA-3′	5′-TCGATCTCAGTGGTATTTGTGA-3′

For both AvBDs and 28S rRNA-specific amplification in Taqman reactions, primers and probes were designed using the Primer Express software program (PE Applied Biosystems, Foster City, CA) (Table 2.5). All AvBD probes were designed to lie across intron-exon boundaries, according to the sequence of the relevant genes, to avoid any amplification from genomic DNA contaminants in the RNA samples.

Table 2.5: Real-time RT-PCR probes and primers. [a]P; Probe, F; Forward primer, R; Reverse primer. [b]Genomic DNA sequences.

RNA target		Probe/primer sequence[a]	Exon boundary	Accession no.[b]
AvBD1	P	5′-(FAM)-ATCCTGCAGCACCCTGGGCCA-(TAMRA)-3′	1/2	AY621315
	F	5′-TGCTCCTCCCCTTCATCCT-3′		
	R	5′-GAAAACAATCTGACTTCCTTCCTAGAG-3′		
AvBD2	P	5′-(FAM)-CCAGGTTTCTCCAGGGTTGTCTTCGC-(TAMRA)-3′	1/2	AY621317
	F	5′-CCTGCTTTTCTCTCTCCTCTTCCT-3′		
	R	5′-CCCTCCTTTACAGAAGAGCATGT-3′		
AvBD3	P	5′-(FAM)-TGGCAGTTCCTGCAGCACCCTG-(TAMRA)-3′	1/2	AY621318
	F	5′-CATCCCCTTCTTCCTCTTGTTTC-3′		
	R	5′-CACGACAGAATCCTCCTCTTATTCT-3′		
AvBD4	P	5′-(FAM)-GAACGGGAAAAGCCCACAGCTCCA-(TAMRA)-3′	1/2	AY621319
	F	5′- TGCTCCTCTTTGTGGCAGTTC-3′		
	R	5′- TCCCGCGATATCCACATTG-3′		
AvBD5	P	5′-(FAM)-CAGCCCTGGTTCTGCCCGGA-(TAMRA)-3′	1/2	AY621320
	F	5′-AGATCCTGCCTCTCCTCTTTGC-3′		
	R	5′-CCCACGGCGCTCACAGT-3′		
AvBD14	P	5′-(FAM)- CCCAGGCTGCACCAGAGTCGGA -(TAMRA)-3′	1/2	AM402953
	F	5′- CTGTTTCTTGTTCTCCTGGCAGTA -3′		
	R	5′- CTTCATCTTCCGACATGTGACAGT-3′		
28S	P	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′		X59733
	F	5′-GGCGAAGCCAGAGGAAACT-3′		
	R	5′-GACGACCGATTGCACGTC-3′		

2.2 Polymerase Chain Reaction (PCR)

Genomic DNA (1 μg/μl) from line N and 6_1 chickens was diluted 1:5 for PCR reactions. cDNA from HD11 macrophage-like cells, peripheral blood monocyte-derived macrophages from 1-week-old line 7_2 chickens, and spleen and lung from 7-week-old line N chickens was used to obtain defensin cDNAs. Defensin primers (Table 2.4) were diluted 1:10 in sterile water from a stock solution of 100 pmol/μl. The enzyme *Taq* polymerase was used at 2.5 units/μl (Promega). Reactions were set up by adding 2 μl of cDNA, 2.5 μl of 10x Buffer (proprietary formulation supplied at pH 8.5, magnesium free; Promega), 1.5 μl of $MgCl_2$ (25 mM), 2.5 μl of dNTPs (10 mM), 1 μl of each primer and 0.5 μl of Taq polymerase in a final reaction volume of 25 μl. Initial denaturing of the DNA was carried out at 94°C for 4 min; this was followed by 30 cycles of denaturing at 94°C for 1 min, annealing of primers at an appropriate temperature for 1 min and new DNA synthesis at 72°C for 1 min. The synthesis was then completed by a final cycle at 72°C for 7 min.

The proofreading enzyme *Pfu* polymerase was used at 2 units/μl (Promega). Reactions were set up by adding 2 μl of cDNA, 5 μl of 10x Buffer (Promega), 1 μl of dNTPs (10 mM), 1 μl of each primer and 0.5 μl of *Pfu* polymerase in a final reaction volume of 50 μl. Initial denaturing of the DNA was carried out at 95°C for 5 min; this was followed by 30 cycles of denaturing at 94°C for 30 sec, annealing of primers at an appropriate temperature for 30 sec and new DNA synthesis at 72°C for 2 min. The synthesis was then completed by a final cycle at 72°C for 5 min.

2.3 Gel electrophoresis

DNA products were separated on a 2% agarose gel (1 g agarose dissolved in 50 ml 1X TAE buffer) while the genomic DNA of AvBD 3 was separated on a 1.5 % agarose gel (0.75 g agarose dissolved in 50 ml 1X TAE buffer) with ethidium

bromide (0.5 μg ml^{-1}) as the staining agent. 50X TAE buffer is composed of 2 M Tris, 50 mM EDTA pH 8.0 and 6% (vol vol^{-1}) glacial acetic acid.

The samples were electrophoresed at 100 V for 30 min (Bio-Rad V.H-N Elektrophorese, UK) and DNA bands were observed under ultraviolet light using an Epi Chemi II Darkroom (UVP Laboratory Products, UK). The samples were prepared by adding 3 μl of loading dye (GelPilot; Qiagen- proprietary formulation), containing bromophenol blue, xylene cyanol and orange G, to 5 μl of sample. A 100 bp DNA ladder (Promega) was used as marker.

2.4 Gel extraction

Gel extraction was carried out using the QIAquick gel extraction kit (Qiagen). The DNA fragment was excised from the agarose gel with a clean and sharp scalpel, the gel slice weighed in a colourless tube and an appropriate volume of buffer QG added (3 volumes to 1 volume of gel). Buffer QG (proprietary formulation), containing guanidine thiocyanate, solubilizes the agarose gel slice and provides the appropriate conditions for binding of DNA to the silica membrane. Once the gel had dissolved completely after incubation at 50°C for 10 min, the sample was applied to a QIAquick column and centrifuged for 1 min at 16,000 x *g*. The flow-through was discarded and 0.75 ml of buffer PE (proprietary formulation), containing ethanol, was added to wash the column. The sample was centrifuged for 1 min at 16,000 x *g*, the flow-through discarded and an additional 1 min centrifugation at 17,900 x *g* was carried out to remove residual ethanol. The column was then placed into a clean 1.5 ml microcentrifuge tube and the DNA eluted by adding 30 μl of sterile distilled water to the centre of the column membrane followed by centrifugation for 1 min at 17,900 x *g*.

2.5 Plasmid extraction

Plasmid DNA was extracted using a QIAprep miniprep kit (Qiagen). An overnight culture of transformed cells (10 ml) was centrifuged 10 min at 1800 x *g*. The pellet was resuspended into 250 µl of buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing RNase A (100 µg/ml; 7000 U/ml) and transfered to a microcentrifuge. Buffer P2 (200 mM NaOH, 1% SDS (w/v)) (250 µl) was then added to the mixture. The NaOH/SDS in the presence of RNase A produces bacterial lysis under alkaline conditions. The SDS solubilizes the phospholipids and protein components of the cell membrane, while the alkaline conditions denature the chromosomal and plasmid DNA, in addition to proteins. The lysate is then neutralised by adding 350 µl of buffer N3 (proprietary formulation), containing guanidine hydrochloride and acetic acid, which gives high-salt binding conditions and causes the precipitation of denatured components except the plasmid DNA that renatures correctly and stays in solution.

The mixture was centrifuged for 10 min at 16,000 x *g* to precipitate all the cell debris. The supernatant was applied to the QIAprep column and centrifuged for 1 min at 16,000 x *g*. The flow-through was discarded and 0.75 ml of buffer PE (proprietary formulation), containing ethanol, was added to wash the column. The sample was centrifuged 1 min at 16,000 x *g*, the flow-through discarded and an additional centrifugation for 1 min at 16,000 x *g* was carried out to remove residual ethanol. The column was then placed into a clean 1.5 ml microcentrifuge tube and the DNA eluted by adding 30 µl of sterile distilled water to the centre of the column membrane and centrifuging for 1 min at 16,000 x *g*.

2.6 PCR purification

DNA purification after a PCR reaction was carried out using a QIAquick PCR purification kit. Five volumes of Buffer PB (proprietary formulation), containing guanidine hydrochloride and isopropanol, were added to 1 volume of the PCR sample, allowing the efficient binding of products as small as 100 bp and removal of primers up to 40 bp. The mixture was added to the column and centrifuged for 1 min at 16,000 x *g*. The flow-through was discarded and 0.75 ml of buffer PE, containing ethanol, was added to wash the column. The sample was centrifuged for 1 min at 16,000 x *g*, the flow-through discarded and an additional centrifugation for 1 min at 16,000 x *g* was carried out to remove residual ethanol. The column was then placed into a clean 1.5 ml microcentrifuge tube and the DNA eluted by adding 30 µl of sterile distilled water to the centre of the column membrane and centrifuging for 1 min at 16,000 x *g*.

2.7 Reverse transcriptase reaction

cDNA was obtained from tissue RNA using a reverse transcription system (Promega). RNA (1 µg) was incubated for 10 min at 70°C to obtain linear RNA for optimal annealing of primers. The mixture was then placed on ice and the reaction was set up by adding 2 µl reverse transcription 10x buffer (100 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100), 4 µl $MgCl_2$ (25 mM), 2 µl dNTP mixture (10 mM), 0.5 µl Rnasin Ribonuclease inhibitor, 1 µl Oligo(dT) primer and 1 µl AMV Reverse Transciptase (15 U/µl) in a final volume of 20 µl. The mixture was then incubated at 42°C for 15 min for the reverse transcription. The reaction was then heated at 95°C for 5 min and incubated at 5°C for 5 min to inactivate the AMV Reverse Transcriptase.

2.8 Cloning inserts

2.8.1 Cloning inserts in TOPO vector

PCR products were cloned using a TOPO TA Cloning kit (Invitrogen). PCR product (4 µl) was mixed gently with 1 µl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and 1 µl of TOPO vector and incubated at room temperature for 30 min. The TOPO cloning reaction mixture (2 µl) was then mixed gently with 30 µl of One Shot *E. coli* TOP10F', and incubated for 30 min on ice. The cells were then heat-shocked for 30 sec at 42°C, mixed with 250 µl of SOC medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated for 2 h at 37°C with shaking. Finally, 50 µl cells were spread on LB agar plates containing 50 µg/ml of ampicillin, 40 µl 40 mg/ml X-gal and 40 µl of 100 mM IPTG, and incubated overnight at 37°C. Any white colonies were tested by PCR with gene-specific primers and an M13 (-40) primer, and analysed on agarose gels at an appropriate percentage to check the presence and the size of the cloned insert. The positive clones were cultured overnight in LB medium containing 50 µg/ml of ampicillin. Glycerol stocks of positive clones were then prepared and stored at -80°C.

2.8.2 Cloning inserts in the expression vector pTRiEx-1.1

All digestions were carried out in 0.5 ml tubes with 20 units of each restriction enzyme: *NcoI*/*XhoI*, *NcoI*/*BglII*, *BsaI*/*XhoI* or *BsaI*/*BglII* (NEB), 2 µl of the corresponding 10x buffer (NEB), 3 µl of plasmid DNA (10 ng/µl) or 1 µl of pTriEx vector (50 ng/µl), made up to 20 µl with sterile water. The reaction was then carried out for 2 h at 37°C and the digestion products run on an agarose gel. The inserts were purified using a QIAquick gel extraction kit, while the pTriEx-1.1 vector was purified with a QIAquick PCR Purification Kit.

The cDNA was then ligated to the pTriEx-1.1 vector in the following reaction: 1 µl of pTriEx-1.1 digested vector, 10 µl of digested cDNA, 1.5 µl of 10x buffer ligase (Promega), 1 µl of T4 DNA ligase and 1.5 µl of sterile water. A control without cDNA was included and all samples were incubated overnight at 16°C.

E. coli DH5α competent cells were produced chemically. Bacteria were grown overnight (37°C, 165 rpm) in 10 ml of LB containing no antibiotics. This culture was diluted 1/100 in 250 ml of fresh LB which was then grown at 37°C (165 rpm) until the OD_{600} reached 0.4-0.6. The culture was transferred into two 300 ml bottles (GSA rotor) and centrifuged (5 min, 6000 x *g*). The pellet was gently resuspended in 1:25 of the original culture volume of ice-cold TFB1 (30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl and pH adjusted to 5.8) and incubated at 4°C on ice for 5 min. The bacteria were again centrifuged (5 min, 6000 x *g*) and resuspended gently in 1:25 of the original culture volume of ice-cold TFB2 (10 mM MOPs pH 6.5, 75 mM $CaCl_2$, 10 mM RbCl, 15% glycerol and pH adjusted to 6.5), incubated on ice for a further 15-60 min and dispensed into 200 ml aliquots which were flash-frozen on dry ice and then stored at -70°C until required.

The competent cells were then transformed with the ligation product. *E. coli* DH5α cells (50 µl) were mixed with 2 µl of ligation product. The cells were incubated on ice for 30 min, heat-shocked at 42°C for 30 sec and returned to ice for a further 2 min. SOC medium (250 µl) was then added to the reaction tube, the vials shaken (200 rpm) for 1 h at 37°C and then placed on ice until required. For each transformation, 20 µl and 40 µl of medium plus cells were plated onto two separate plates of LB agar containing 50 µg/ml of ampicillin and the plates were incubated at 37°C overnight.

2.9 Sequencing on the CEQ 8000 Sequencer

Reactions were set up with 1 µl plasmid (200 ng), heat-treated for 1 min at 96°C in 13 µl of distilled water, then 1 µl of M13 (-40) forward primer, 4 µl of quickstart and 1 µl of dilution buffer from CEQ DTCS Quick Start Kit (Beckman Coulter, CA, USA) were added. Initial denaturing of the DNA was carried out at 96°C for 2 min. This was followed by 30 cycles of denaturing at 96°C for 20 sec, annealing of primers at 50°C for 20 sec and new DNA synthesis at 60°C for 2 min. Following the cycle sequencing, 5 µl of a stop solution (2 volumes of 100 mM EDTA, 2 volumes of 3 M sodium acetate pH 5.2 and 1 volume of glycogen at 20 mg/ml) were added to each sample. Ice-cold 95% ethanol (60 µl) was added to the samples and they were centrifuged at 16,000 x *g* for 15 min to precipitate the DNA. The pellets obtained were washed twice with 200 µl of ice-cold 70% ethanol and air-dried for 1 h. Finally, 40 µl of Sample Loading Solution from CEQ DTCS Quick Start Kit (Beckman Coulter, CA, USA) was added to each sample with a drop of mineral oil to stabilise the reaction and they were loaded on the CEQ 8000 Sequencer (Beckman Coulter).

2.10 Plasmid purification using a QIAGEN plasmid Midi kit

Plasmid purification was carried out by inoculating 25 ml medium with a starter culture diluted at 1/500 and incubating at 37°C for 12 h with vigorous shaking. The cells were centrifuged at 6000 x *g* for 15 min at 4°C and the pellet resuspended into 4 ml of buffer P1, containing RNase A. Buffer P2 (NaOH/SDS) (4 ml) was then added to the mixture. The NaOH/SDS in the presence of RNase A produces bacterial lysis under alkaline conditions. The SDS solubilises the phospholipids and protein components of the cell membrane, while the alkaline conditions denature the

chromosomal and plasmid DNA, in addition to proteins. The lysate was then neutralised by adding 4 ml of buffer P3 (3 M potassium acetate, pH 5.5), which gives high-salt binding conditions and causes the precipitation of denatured components, except the plasmid DNA that renatures correctly and stays in solution. The mixture was centrifuged at 20,000 x *g* for 30 min at 4°C to precipitate all the cell debris. The supernatant was again centrifuged at 20,000 x *g* for 30 min at 4°C to eliminate suspended or particulate material that can clog the QIAGEN-tip. The supernatant was applied to a QIAGEN-tip 100, previously equilibrated with 4 ml of buffer QBT (pH 7.0). The QIAGEN-tips contain an anion-exchange resin that interacts with the negatively charged phosphates of the DNA plasmid via positively charged DEAE groups present in the resin surface. The binding, washing and elutions steps are therefore strongly influenced by pH. The column was washed twice with 10 ml of buffer QC (1 M NaCl, 50 mM MOPS,pH 7.0, 15% isopropanol (v/v)) and DNA was finally eluted with 5 ml of Buffer QF (1.25 M NaCl, 50 mM Tris-HCl, pH 8.5, 15% isopropanol (v/v)). Finally, the plasmid DNA was precipitated with 3.5 ml of isopropanol, centrifuged at 15,000 x *g* for 30 min at 4°C, washed with 2 ml of 70% ethanol and centrifuged again at 15,000 x *g* for 10 min. The pellet was air-dried for 10 min and redissolved in 1 ml of sterile water.

2.11 Expression of defensin mRNA in COS-7 cells

The COS-7 cell line is an African green monkey kidney cell line, derived from the CV-1 cell line by transformation with an origin-defective mutant of SV40. The cells were cryopreserved at 1.5 x 10^6 cells/ml after 6 passages and thawed in a water-bath at 37°C. They were then washed with COS-7 cell growth medium (360 ml DMEM medium, 200 mM L-glutamine, 4.5 g/l glucose, 110 mg/l sodium pyruvate,

3.7 g/l sodium bicarbonate) containing 10% foetal calf serum (FCS) and incubated at 37°C in 5% CO_2 in a T75 flask for 4 days. The cell layer was washed twice with pre-warmed PBS (12 g/l PBSa powder, BioWhittaker) and detached from the flask after 5 min incubation at 41°C with ~5 ml of trypsin (0.25% trypsin in PBS)/versene (9.95 g PBSa powder, 0.2 g EDTA) solution at a 1:10 dilution. The trypsinisation was stopped with ~15 ml of growth medium containing FCS and the cells pelleted at 900 x *g* for 5 min to determine the viable cell concentration using a haemocytometer. T75 flasks were seeded with 7.5 x 10^5 cells in 15 ml of growth media and incubated at 37°C in 5% CO_2 for 2 days. The trypsinisation and viable cell count procedures were repeated until the COS-7 cell concentration was sufficient to seed the appropriate number of T25 flasks with 2 x 10^6 cells each to carry out the transfection procedure. T25 flasks with 2 x 10^6 cells were incubated at 37°C in 5% CO_2 for 24 h and washed twice with PBS. To each T25 flask was added 5 ml of serum-free growth medium containing 37.5 µg DNA, 50 µl of chloroquine and 30 µl of DEAE/dextran and incubated for 3 h at 37°C in 5% CO_2. The cells were washed with PBS and 10% DMSO was added for 2 min. The mixture was then removed, replaced with 5 ml growth medium containing 10% FCS and the T25 flasks were finally incubated for 24 h at 37°C in 5% CO_2. The growth medium was changed for serum-free medium and cells were harvested 3 days post-transfection to obtain defensin mRNA using an RNeasy kit (QIAGEN).

2.12 Experimental plan

All birds were from specified-pathogen-free (SPF) inbred flocks reared in the Institute for Animal Health (IAH) experimental animal house. Line 6_1 and N chickens were inoculated orally at one day of age with 0.1 ml of gut flora to avoid

the development of different flora in the different lines. The gut flora was obtained from the caecal contents of an adult out-bred SPF chicken from the IAH flocks, incubated statically overnight in 10 ml LB broth.

In a first experiment, non-infected chickens from each line (n = 3) were killed at 6 weeks of age by cervical dislocation and the following tissues were collected for quantification of AvBD mRNA levels: lung, bursa, bone marrow, jejunum, ileum, caeca and caecal tonsil.

In a second experiment, non-infected chickens from each line (n = 3) were killed at 6 and 7 weeks of age as controls. Housed in a separate room, a group of 10 birds from each line, reared in the same cage, were infected orally at 6 weeks of age with 0.1 ml of *S.* Typhimurium F98 overnight culture and killed after 1, 2 and 7 days post-infection (n = 3 for each-time point). These time points were chosen according to the bacteriological results previously obtained by Barrow P. A. *et al*, 2004. A similar experiment was done in parallel with chickens infected with *S.* Gallinarum 9 (n = 5 for each-time point). The caecal contents and caecal tonsils were collected for quantification of bacterial load and AvBD mRNA levels respectively.

In a third experiment, 27 chickens from each of line 6_1 and N were housed in separate cages in the same room. Every week for nine weeks, 3 birds from each line were killed to collect caeca and caecal tonsils for quantification of AvBD mRNA levels.

Finally, in a fourth experiment, designed and performed by Dr P. Wigley and Ms L. Chappell, 60 line 7_2 chickens were housed in separate cages in the same room. Twenty chickens were then infected orally at 1 week of age with 0.1 ml of *S.* Pullorum 449/87 overnight culture and killed after 24 h, 1, 2 and 4 weeks post-infection (n = 5 for each time-point). A similar experiment was done in parallel with

chickens infected with *S.* Enteritidis p125109 (n = 5 for each time-point) and 20 other birds were inoculated with 0.1 ml of LB broth as controls. The caecal tonsils and spleen were subsequently analysed for AvBD mRNA levels.

2.13 Bacterial enumeration

Bacterial load was assessed as previously described (Barrow *et al.*, 2004). Caecal contents were plated on Brillant Green agar containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml). For quantitative enumeration, caecal contents were diluted and homogenised in phosphate-buffered saline (PBS). The viable count of *Salmonella* in the samples was estimated by plating aliquots of ten-fold dilutions onto selective Brillant Green agar. The plates were finally incubated at 37°C for 24 h.

2.14 RNA extraction

2.14.1 From COS-7 cells

Cells were harvested after trypsinisation and mixed with 600 μl of buffer RLT (proprietary formulation), containing guanidine thiocyanate and 0.01% β-mercaptoethanol (v/v), which lyses the cell membrane. The lysate was homogenised with a QIAshredder spin column and centrifuged for 2 min at 16,000 x *g*. One volume of 70% ethanol was added to the supernatant for optimal absorption of RNA to the RNeasy mini column. The sample (700 μl) was applied to the column and centrifuged for 15 sec at 16,000 x *g*. The flow-through was discarded, 700 μl of buffer RW1 (proprietary formulation), containing ethanol, added to the column, and centrifuged for 15 sec at 16,000 x *g* to remove contaminants. The collection tube was removed with the flow-through and the column placed into a new collection tube to wash the column with 500 μl of buffer RPE (proprietary formulation). The tube was

centrifuged for 15 sec at 16,000 x *g*, another 500 µl of buffer RPE added to the column and finally centrifuged for 2 min at 16,000 x *g* to dry the RNeasy silica-gel membrane and obtain high quality RNA, which was eluted in 30 µl of water.

2.14.2 From tissues

In order to obtain total RNA, tissues were immediately stabilized in RNA*later* RNA Stabilisation Reagent (Ambion, Huntingdon, UK) and stored at -20°C until extraction of RNA using an RNeasy Kit (Qiagen, Crawley, UK) After thawing the samples, 30 mg of tissues were mixed with 600 µl of buffer RLT, containing β-mercaptoethanol, which lyses the cell membrane. A Mixer Mill MM 300 (Retsch GmbH & Co., Germany) was then used to disrupt tissue and homogenize the lysate for 4 min at 20 Hz. The tissue lysate was centrifuged for 3 min at 16,000 x *g* to pellet the cell debris and the supernatant was transferred into a new microcentrifuge tube. One volume of 70% ethanol was added to the supernatant for optimal absorption of RNA to the RNeasy mini column. The sample (700 µl) was applied to the column and centrifuged for 15 sec at 16,000 x *g*. The flow-through was discarded, 700 µl of buffer RW1 added to the column, and centrifuged for 15 sec at 16,000 x *g* to remove contaminants. The collection tube was removed with the flow-through and the column placed into a new collection tube to wash the column with 500 µl of buffer RPE. The tube was centrifuged for 15 sec at 16,000 x *g*, another 500 µl of buffer RPE was added to the column and finally centrifuged for 2 min at 16,000 x *g* to dry the RNeasy silica-gel membrane and obtain high quality RNA, which was eluted in 30 µl of water. The samples were stored at -20°C until quantification by real-time RT-PCR.

2.15 Optimising primer concentrations for TaqMan assays

Optimisation was carried out with an ABI Prism 7700 Sequence Detector in a Thermofast 96-well plate. Firstly, 19 µl of master mix, containing 1x Master mix (proprietary formulation, Eurogentec S.A., Belgium), 0.25 units/µl Moloney Murine leukaemia virus reverse transcriptase and 0.1 units/µl RNase inhibitor, 0.1 µM probe and the remainder of the volume made up with RNase-free water, were added to each well. Each primer (1 µl), diluted to obtain the following final concentrations, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 µM, was added to the appropriate wells. Finally, 5 µl of RNA samples, previously prepared with serial ten-fold dilutions from 10^{-1} to 10^{-6} from a stock concentration around 2 mg/ml, were added to the wells so that each dilution of RNA sample was added to the wells containing the different primer concentrations. In addition, 5 µl of RNase-free water was added to six no template control wells. The RT-PCR conditions were set up with an initial step at 50°C for 2 min to prevent the amplification of contaminants, followed by the reverse transcriptase step at 60°C for 30 min. The following PCR activated the Ampli Taq polymerase at 95°C for 5 min, followed by 40 cycles to denaturize and extend the DNA respectively at 94°C for 20 sec and 59°C for 1 min (see Appendix 3).

2.16 Quantification of AvBD mRNA expression by real-time quantitative RT-PCR

AvBD mRNA expression in tissues from chickens uninfected or infected with *S.* Typhimurium and *S.* Gallinarum was quantified using real-time quantitative RT-PCR as previously described (Kaiser *et al.*, 2000; Kaiser *et al.*, 2002; Kogut *et al.*, 2003; Swaggerty *et al.*, 2004; Swaggerty *et al.*, 2006). Primer and probe sequences are shown in Table 2.5.

Real-time quantitative RT-PCR was performed using the Reverse Transcriptase qPCR Master Mix RT-PCR kit (Eurogentec, Seraing, Belgium). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) with the following cycle profile: one cycle of 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min, and 40 cycles of 94°C for 20 sec, 59°C for 1 min or the 7500 Fast Real-Time PCR System (PE Applied Biosystems, Foster City, CA, USA) with the following cycle profile: one cycle of 50°C for 2 min, 60°C for 15 min, and 95°C for 5 min, and 40 cycles of 94°C for 20 sec, 59°C for 1 min. Quantification was based on the increased fluorescence detected due to hydrolysis of the target-specific probes by the 5′-exonuclease activity of the *rTth* DNA polymerase during PCR amplification (see Appendix 4). The passive reference dye 6-carboxy-c-rhodamine, which is not involved in amplification, was used for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in the reporter dye passes a significance threshold (ΔR_n)

To account for variation in sampling and RNA preparation, the Ct values for AvBD-specific product for each sample were standardised using the Ct value of 28S rRNA product for the same sample. To normalise RNA levels between samples within an experiment, the mean Ct value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA Ct values about the experimental mean were calculated. The slope of the 28S rRNA $\log_{10}$ dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective AvBD $\log_{10}$ dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust AvBD-specific Ct values, as follows:

Corrected Ct value = Ct + (Nt-Ct')* S/S' where Ct = mean sample Ct; Nt = experimental 28S mean; Ct'= mean 28S of sample; S = AvBD slope; S'= 28S slope. Results were then expressed as 40-Ct values.

2.17 Expression of AvBDs in Baculovirus system

AvBDs were expressed using a baculovirus system at the University of Reading in Prof Ian Jones' lab (see Appendix 5). Bacmid DNA (500 ng) was linearised by digesting at the unique *Bsu*36I site with 1 µl of *Bsu*36I (NEB), 2 µl 10x NEB buffer 3 and 0.2 µl 100x BSA (10 mg/ml) in a final reaction volume of 20 µl, incubating at 37°C for 3 h followed by heat inactivation of the enzyme at 80°C for 20 min. For the transfection, 500 ng of transfer vector DNA, AvBD cDNA cloned into pTriEx1.1, was mixed with 500 ng bacmid and 12 µl of lipofectin, diluted 2:1 (Invitrogen), in a final reaction volume of 25 µl and incubated at 25°C for 30 min. Sf9 insect cells at 10^6 cells/well were incubated for 1 h at 28°C to allow the cells to adhere. Insect-Xpress medium (BioWhittaker, Walkersville, MD) with 2% FCS and 2% penicillin-streptomycin was then removed, replaced with the DNA mixture/lipofectin previously mixed with serum-free media and incubated at 28°C. After 24 h incubation, serum-free media was replaced with 2% FCS media and the cells were incubated at 28°C for 4 days. The cells were finally centrifuged for 10 min at 16,000 x *g* and the supernatant transferred to a new eppendorf tube and stored at 4°C. This contains low titre recombinant baculovirus, named P0.

The recombinant baculovirus P0 was then amplified by infecting 15 x 10^6 cells per T150 tissue culture flask with 1 ml of recombinant virus P0 mixed with 10 ml of media with 2% FCS, and incubated at 28°C for 30 min. Media (19 ml) containing 2% FCS were added and the cells incubated for 1 week at 28°C. The cells were finally

centrifuged for 20 min at 5,000 x *g* and the supernatant transferred to a new eppendorf tube and stored at 4°C. This contains high titre recombinant baculovirus, named P1.

Finally, small and large-scale production of AvBDs was carried out. The small-scale production was performed by infecting 1 x 10^6 cells per well in a 6-well plate with 250 µl of recombinant virus P1 mixed with 250 µl of serum and antibiotic-free medium and incubated at 25°C for 1 h. The large-scale production was carried out by infecting 15x10^6 cells per T150 tissue culture flask with 500 µl of recombinant virus P1 mixed with 9.5 ml of media with 2% FCS and incubated at 25°C for 1 h. The recombinant virus P1/media was then removed and changed to serum- and antibiotic-free media for large and small-scale production respectively. After 3 days at 28°C, the cells were finally centrifuged for 20 min at 5,000 x *g* and both supernatant and pellet were stored at -20°C until purification.

2.18 Analysis of baculovirus DNA

To prepare the DNA template, the virus is lysed and treated with proteinase K. For this purpose, 10 µl of virus stock were mixed with 89 µl of lysis buffer (10 mM Tris-HCl, pH 8.3, 100 µg/ml of gelatine, 0.45% TritonX-100, 0.45% Tween 20 and 50 mM KCl) and 1 µl proteinase K (6 mg/ml in water) added last and mixed with a pipette tip. The mixture was incubated for 1 h at 60°C then for 10 min at 95°C to heat-inactivate the proteinase K. Finally, the product obtained was used as a template for PCR reactions with AvBD-specific primers.

2.19 Purification

2.19.1 Cationic exchange chromatography

For small-scale purification, 5 ml of supernatant were mixed with 5 ml of start buffer and the cell pellet was mixed with 4 ml of start buffer and 80 µl of inhibitor cocktail tablet (ROCHE), sonicated 10 min and made up to 10 ml with start buffer. The start buffer is the buffer used in the first step of ionic purification and described in the each figure legend. For large-scale purification, 30 ml of supernatant were mixed with 20 ml of start buffer and the pH adjusted to the pH noted in the figure legend for each experiment. Finally, the samples were centrifuged at 15000 x *g* for 15 min at 4°C. The start buffer was either composed of 50 mM bicine or 32 mM ammonium acetate.

A 5 ml HiTrap SP HP (Amersham Biosciences) cationic exchange column, used for small-scale purification, was placed on the FPLC system, washed with filter-sterilised distilled water and equilibrated with 2 x column volumes of start buffer. The samples were applied to the cationic column and washed with 5 column volumes of start buffer at a 5 ml/min flow rate. The elution was then carried out with a continuous ionic gradient of 0 to 0.5 M NaCl (start buffer, 0.5mM NaCl) at 5 ml/min flow rate and a gradient volume of 10 x column volumes. The eluate was collected in 2 ml fractions and those containing the elution peak were mixed and further purified by RP-HPLC.

For large-scale purification, a 25 ml CM Sepharose™ Fast Flow (GE Healthcare) cationic exchange column was placed on the FPLC system, washed with filter-sterilised distilled water and equilibrated with 3 x 50 ml of start buffer. The samples were applied to the cationic column and washed with 2 x 50 of start buffer at a 20 ml/min flow rate. The elution was a one step elution carried out with 2 x 50 ml of

elution buffer (start buffer, 0.8 M NaCl) at a 10 ml/min flow rate. The eluate was collected in 5 ml fractions and those containing the elution peak were mixed and further purified with RP-HPLC.

2.19.2 Reverse Phase-HPLC

Reversed phase separations were performed by loading the products of the ion exchange chromatography onto a 4.6 x 250mm Vydac C18 column (Biocad Sprint HPLC, Perceptive Biosystems, Cambridge, USA). The separations were achieved using the following gradient: 2 min of 0% solvent B, 20 min gradient from 0 to 80% of solvent B then a 4 min gradient from 80 to 0% of solvent B and finally a 1 min step of 0% solvent B. Solvent A was composed of 0.1% TFA in distilled water versus solvent B composed of 0.1% TFA in acetonitrile.

2.20 Tris-Tricine SDS-PAGE

Samples were mixed with SDS loading buffer (200 mM Tris-HCl, pH 6.8, 2% SDS, 40% glycerol and 0.04% Coomassie Brilliant Blue), boiled for 10 min and centrifuged at 16,000 x *g* for 1 min. A 16.5% Tris-Tricine SDS-PAGE Ready gel (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) (Schägger & von Jagow, 1987) was placed in an electrophoresis tank, which was then filled with 10x Tris/Tricine/SDS Buffer (100 mM Tris, pH 8.3, 100 mM Tricine, 0.1% SDS) diluted to 1x. The sample mixtures were then loaded into the SDS gel and one well was used to load the marker, Precision Plus Protein standards (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The gel was then run for 90 min at 65 mA. SDS gels were then Coomassie Brillant Blue or silver stained.

2.20.1 Coomassie Blue staining

The proteins were first fixed by incubating the gel in 5 gel volumes of fixing solution (50% methanol, 10% acetic acid) for 2 h at 25°C with gentle shaking. The fixing solution was then replaced with Coomassie Brillant Blue (CBB) staining solution (0.25 g of CBB R250 in 45% methanol and 10% acetic acid) and incubated for 4 h at 25°C with gentle shaking. The gel was rinsed in fixing solution and destained in 5 gel volumes with destaining solution (30% methanol, 10% acetic acid) for 4-8 h, changed 3-4 times during this period. Finally, the gel was stored in 7% acetic acid and photographed with a digital camera.

2.20.2 Silver staining

Proteins were first fixed by incubating the gel in 5 gel volumes of fixing solution (50% methanol, 12% acetic acid, 0.02% formaldehyde) for 16 h at 25°C with gentle shaking. The gel was then washed twice in 5 gel volumes of 50% ethanol for 30 min at 25°C, pretreated in fresh 0.02% sodium thiosulphate for 1 min, rinsed thoroughly 3 times in distilled water and incubated for 30 min in 5 gel volumes of silver nitrate solution (0.2% silver nitrate, 0.03% formaldehyde in an air-tight, dark bottle) at 25°C with gentle shaking. The gel was rinsed twice in distilled water and incubated for 2-5 min in fresh developing solution (6% sodium carbonate, 0.02% formaldehyde, 0.0005% sodium thiosulphate). Once the desired contrast has been reached, the gel was stored in stop solution (16% acetic acid, 50% methanol) until photographed.

2.21 Antimicrobial assay

An *E. coli* BUE55 (Devine *et al.*, 1999; Moore *et al.*, 1996) overnight culture was diluted ten times in fresh LB media and incubated for 2 h at 37°C. The culture was centrifuged for 10 min at 2000 x *g*, washed with 5 ml of ice-cold PBS and diluted to $2x10^5$ cfu/ml. The bacterial concentration was determined by spectrometry at OD_{620} and calculated with the following formula: cfu/ml = OD_{620} x 2.5 $x10^8$, according to Lehrer *et al.*, (1991). Then, 100 µl of *E. coli* BUE55 were mixed with either an appropriate concentration of human β-defensin 3 (Peptide Institute, Inc., Osaka, Japan), from 20 µg/ml to 1.25 µg/ml, or 50 µl of peak sample and then incubated for 2 h at 37°C. HBD3 was diluted in 50 µl PBS, 50 µl LB, while the samples tested were added to 25 µl PBS, 25 µl LB. In parallel, a control without HBD3 and a second control with 0.01% acetic acid only were also set up and incubated for 2 h at 37°C. The cells were then counted by diluting the cells from 10^{-1} to 10^{-3} in PBS and plating 50 µl of each dilution on two LB plates, which were incubated for 24 h at 37°C. The percentage of killing was determined with the following calculation: % killing = ((cfu *control* - cfu *test*)*100)/cfu *control* (when cfu *test* > cfu *control* = 0%) with cfu = number of cells * dilution factor * 20.

2.22 Mass spectrometry

Samples were loaded onto Waters QTOF Premier from vials in 1 or 5 µl volumes and separated using the manufacturer's own trap, which consisted of a 180 micron x 20 mm 5 micron Symmetry C18 and a reverse phase column of 100 micron x 100 mm BEH130 C18. Gradients were pumped at 350 µl/min by a Waters nano Acquity; 1% solvent B for 1 min, a gradient of 1-50% solvent B for 59 min, 50-85 % solvent B for 1 min then 85-1% solvent B for 1 min. Solvent A was 0.1% TFA in

5% acetonitrile, 95% water, versus solvent B composed of 0.1% TFA in 95% acetonitrile, 5% water.

Chapter 3 - Characterisation of AvBDs 1, 2, 3, 4, 5 and 14 genomic sequences and cloning of their cDNAs

3.1 Introduction

Chicken β-defensins have recently been renamed avian beta-defensins (AvBDs) (Lynn *et al.*, 2007). Like mammalian β-defensins, they are composed of six cysteine residues that form three pairs of disulphide bridges with a triple-stranded β-sheet structure. In 1994, three AvBDs were first isolated from chicken leukocytes; gallinacin 1, gallinacin 1α (also named chicken heterophil peptide (CHP) 1 and CHP 2 respectively) and gallinacin 2 (Evans *et al.*, 1994; Harwig *et al.*, 1994). These antimicrobial peptides inhibited gram-positive and gram-negative bacteria such as *Listeria monocytogenes* and *S.* Enteritidis and the yeast *Candida albicans* (Evans *et al.*, 1995; Sugiarto & Yu, 2004). Determination of the full amino acid sequence of the AvBDs revealed the absence of the negatively charged propiece reported in classical defensins, which is thought to inactivate the positively charged mature sequence allowing the storage of defensins in granules. Its absence in chicken defensins suggested that the chicken uses some other mechanism to neutralize the positive charges of the AvBDs whilst they are stored in granules (Brockus *et al.*, 1998). In 2001, an epithelial β-defensin named gallinacin 3 was also characterised. This β-defensin was expressed in epithelial organs such as the lungs, bursa and intestine (Zhao *et al.*, 2001). Therefore, at the beginning of this project these four AvBDs were selected to study their role in the chicken's innate immune response, along with three, at that time, novel AvBDs characterised in this laboratory, AvBDs 4, 5 and 14. Since 2004, the chicken genome sequence became available and ten novel β-defensins have since been described, including AvBDs 4 and 5 (Higgs *et al.*, 2005; Lynn *et al.*, 2004; Xiao *et al.*, 2004). However, AvBD14 has yet to be described in the literature.

In this chapter, the genomic and evolutionary characteristics of AvBDs, including the novel AvBD14, will be analysed. In addition, the genomic sequences and the cDNAs

of AvBDs 1, 2, 3, 4, 5 and 14 will be characterised. The AvBDs cDNAs were then cloned into an appropriate expression vector, pTriEx1.1, to express their mRNA in COS-7 cells and protein using a baculovirus system.

3.2 Results

3.2.1 Determination of the genomic sequences of AvBDs 1/1α, 2, 3, 4 and 5

In the absence of monoclonal antibodies specific for the AvBDs, in order to characterize their expression in response to microbial infection, real-time quantitative RT-PCR (Taqman) was used to measure mRNA levels. In Taqman assays, either a primer or the probe is designed to cross exon boundaries to specifically amplify mRNA. However, at the start of the project, for gallinacins 1, 1α, 2 and 3 only the mRNA sequences were available. Therefore, specific primers (Table 2.3) were used to amplify the respective sequences from genomic DNA of line N and 6_1 chickens. After optimization of the PCR, the annealing temperature required to amplify AvBD genomic DNA was 58°C. The genomic sequences obtained showed that the AvBD genes were composed of three exons and two introns (Figure 3.1) and the five genes have the exon junctions in the same frame and the same signal peptide lengths (20 amino acids). The third exon was too short to design primers and probes properly, and they were therefore designed across the first exon/intron boundaries using ABI Prism Primer Express software (Table 2.5).

3.2.2 AvBD1 and gallinacin 1α are polymorphic variants of the same gene

The peptide sequences of AvBD1 and gallinacin 1α differ by only three amino acids due to three nucleotide substitutions. The proofreading enzyme *Pfu* was used in PCR and the genomic sequence obtained from the two inbred lines was shown

Figure 3.1: Gene sequences and predicted peptide sequences of AvBDs 1, 2, 3, 4 and 5. For the gene sequences, exons are in upper case and introns in lower case.

AvBD1 (Gal 1α form)

```
ATGCGGATCGTGTACCTGCTCCTCCCCTTCATCCTCCTCCTGGCCCAGGGTGCTGCAGgtgaggtgtgagttctgtggggttctccatat 90
M  R  I  V  Y  L  L  L  P  F  I  L  L  L  A  Q  G  A  A  G
cccaggaggtggcttgtcagggatgggtaacgactaggagggctctgatcagttggttcaggagggagggaagatttaggttggatatca 180

gggggaagttctttacagagagagaggtgaggtgctggaacagctgcccagagaggctgtggatgccccgtccatccctggaggtgttca 270

aggccaggttggatggggccctgggcagcctgggctggtgttagatgtggaggttggtggccctgcctgtggtgggtgggttggagcttc 360

atgatccttgaggtcccttccaacccaaccattctgtgattctgtggtttggatgagtggctgggcttttgggtttggtgctttgtgcac 450

gtgttagactgagatccatgggacagccactctagaaccacacacagcttgtacaggtatcccacactcattttcttttggtctgtgcag 540

GATCCTCCCAGGCTCTAGGAAGGAAGTCAGATTGTTTTCGAAAGAATGGCTTCTGTGCATTTCTGAAGTGCCCTTACCTCACTCTCATCA 630
  S  S  Q  A  L  G  R  K  S  D  C  F  R  K  N  G  F  C  A  F  L  K  C  P  Y  L  T  L  I  S
GTGGGAAATGCTCAAGATTTCACCTCTGCTGCAAAAGgtaagctttggaattagggatgaaattggatctgctaccacgatggcagaaat 720
  G  K  C  S  R  F  H  L  C  C  K  R
agctgttgttgtgtttgatccccaaacctagctactggctttgggctatatatgatccagggcaggggcttggggaggaaaggagaaggt 810

gctaggaccggtcctttaaaggaactggaggaaccccagatcagacactggcctccccattgccctcagttacacggggctgcctggctt 900

gctggtttcacaaatgcttccccagttggtgcagagtggagactctcccctgggtagtgtgaggcacagaacccattccctgatgtctct 990

gcaaaaccttggaaaccaagctgaaaccaagctgtctgctatgcaggctgcttactacctgcattgagattagtgtcaatgtgtcagtgt 1080

tatccaggagaagtgatgcatagtgagagacagaaaaaggagaataaaaagaggtgacctcacagagtgttttcttcctgcagAATATGG 1170
                                                                                    I  W
GGCTGA 1176
G  *
```

AvBD2

```
ATGAGGATTCTTTACCTGCTTTTCTCTCTCCTCTTCCTGGCACTCCAGGTTTCTCCAGgtaagatgaaagaggaattaaaggggaggata 90
M  R  I  L  Y  L  L  F  S  L  L  F  L  A  L  Q  V  S  P  G
acgactgggttatggggaagggtttgcagacccgctttgtgagctcacctttcaacgtggccaaaccctcacagcagtccttaaggcagc 180

tgagtgagtggagctgccttgccttgcagaatcagagggaacttggttgctgttgttgcagGGTTGTCTTCGCCCCGGCGGGACATGCTG 270
                                                                L  S  S  P  R  R  D  M  L
TTCTGTAAAGGAGGGTCCTGCCACTTTGGAGGGTGTCCCAGCCATCTAATCAAAGTCGGAAGCTGCTTCGGGTTCCGTTCCTGCTGCAAA 360
F  C  K  G  G  S  C  H  F  G  G  C  P  S  H  L  I  K  V  G  S  C  S  R  F  Y  L  C  C  K
TGgtgagtttgaccttcactgacgttcatccatcgcgtaagtggacaaatgcattttacccaagatgctgctgaatgttcggtcttggat 450
W
ttatgaaggaaacagtacattacgagggcagcctggtgtaagttgctagtagggctttacagttgtctttctcctgagatgtgctgctga 540

ggtgtacaccatgatgtgtccaggcacaaagggtaaagtatggccatagatgccagccacgtgcagtcccagctctttgcttataagtcc 630

cagcccttatagctcctctgccaggggttttgtattttcagaactgggctgttatggtgcatggggaacaaaagggttgcgctgcaggg 720

tgaacacggatctgagtgcagttgagtctgtgcaaaaagtgaaactgcatcaaaagaaaatctaatgccattgggactgaacgcactcac 810

cccaaggccaggggataccaattcagttccctgcttttcccggagcgatagcaaagcactcctcccagtcagatgggactgcacaaggct 900

gtcccaatccgacttgcatgtgacaataggtattttggaatgtatataaccaagaggaagacgtgcatggattgagagcgagtagggaag 990

gaatgtaaatacaaaaacaatctgatttctttgtctgtttgtgcagGCCTTGGAATGCATAA 1052
                                              P  W  N  A  *
```

AvBD3

```
ATGAAGATCCTGTACCTGCTCATCCCCTTCTTCCTCTTGTTTCTCCAGGGTGCTGCAGgtgagaggggaagatggggtgaggtgtgagcc 90
M  R  I  V  Y  L  L  I  P  F  F  L  L  F  L  Q  G  A  A  G
catatcagtagggtcttccctgttctgggaagaaattgcctttgttggaacaacacagaggttgggaggcaatgacataaattctgtgag 180

tgccccttccaagagatgcagaaacaccccataaatagaagcctggcttggtgtgtgttggaaggagttctggctgcaggttgcaatcct 270

gtgtccagctgctcctctatgggtgtctctgaatgagcacccattggcaaggctgaggtttgggcagcagcaaaacatcataaaaacatt 360

aaggttgtaagaaacctcaaagatcatacagtctaaccatctacctactaccagtattacccgttaaaccatgcccctaagtactacatc 450

tatcctatcataaaacacctccaaggacagtaatcaccacttccctgggcagcctgttctaatgcattaccactctttcagaggtgacct 540

gaacctcccctgggctaaattaacgccattccctcatccttcagtgggacctgtgcagggatgggatagagccataggtggcttttgact 630

cagtgggatgactgagacacaatgctgtcccattttggccagtagtgtctcagctgagtgccccaaagagtccttgccctgagccatgac 720

ctctttgagggttgggtttccatcagaggtcccttgaactctgggtgcagtagtgacaggtatgcaaggagtgccccagttgccagctcc 810

actggcccaggaagaggtggacatgaaggtgggatggacatacaggtggggagcctgcacacgtctccttggagctcctgctggtctttc 900

ccatctgctgtcttgggttcccacactggtgtgaaagaatccttctaggtgagacaccttgctctgaaagacccaaatattagtctctgc 990

catccatttttacaatatattctccctgtatgactttccatgtaccagGAACTGCCACCCAGTGCAGAATAAGAGGAGGATTCTGTCGTG 1080
                                                  T  A  T  Q  C  R  I  R  G  G  F  C  R  V
TTGGGAGCTGCCGCTTCCCACACATAGCTATTGGGAAATGTGCAACATTTATTTCCTGCTGTGGAAGgtaagatctggattcctggctga 1170
  G  S  C  R  F  P  H  I  A  I  G  K  C  A  T  F  I  S  C  C  G  R
gaaaagggatccctcctttgcctattgaaatagctgtcatacatctctctctcgccaacatctagcaggcaatatctggcatatgtttgg 1260

actagatgatcttagaggtcttttccaacctacatgtttctatgataaatgcaccacaagaagcccaggaagggaaaagccctcgtgggt 1350

ttggaggagccctgtgtgaggctgaaggaaccccatgctcagtcagcagccatccattcttcttagagtcccactcatcatttgcagggg 1440

gatctcccaggattggagatgatcagggatgttgtcatagaatcatagaattgctaaggttggaaaagacccacaggatcatccagtcca 1530

accattcgcccttcaccaatggttctcgctaaaccatgtccctcaacacaacatccaaacgctctttgaacaccaccaggctcggtgact 1620

ccaccacctctctgggcagcccactgcagtgcctgaccacccttcagacaagtattatttcctaacgtccagcctgaaccttccctggt 1710

gcagcttgaagccattccctctagtcctatcactgtcacccaagagaagaggccgacccccagctccctacaacctcccttcaggtagtt 1800

atagagagcaataaggtctcccctgagcctcctcttctccagactgaacaatcccagctccttcagcggctcctcataatgtttgtgatg 1890

ttgctgcaaagacctggactgagggctagcaccactaatcggagcatgagttccaataagccatgagtggtaagggctggagttaccctt 1980

tgaacattgacaggggaggtttaggttggatattaggaagaagcttttcacccagagggtggtgatgcactgaacaggttgcccaaggag 2070

gctgtggatgccccatccctggaggcattcaaggccaggctggatgtggctctgggcagcctggtctgctggttggcgaccctgcacata 2160

gcaggggttggaactggatgatcactgtggtccttttcaacccaggccgttctatgattctaaaattcaatcaggtcccaaggcttgtt 2250

tgtccctggagaggagatgagagagcagggagaagcgagttgcatgcaggtgacacacttgtctgttttctctgtagAGCATATGAGGTT 2340
                                                                                A  Y  E  V
GATGCCCTGAATTCTGTGAGGACATCGCCCTGGCTTCTCGCTCCTGGAAACAACCCCCATTGA 2403
D  A  L  N  S  V  R  T  S  P  W  L  L  A  P  G  N  N  P  H  *
```

AvBD4

```
ATGAAAATCCTTTGCTTTTTCATCGTGCTCCTCTTTGTGGCAGTTCATGGAGCTGTGGgtaaggagtaagtgaaagcgtgaggctgtata 90
M  K  I  L  C  F  F  I  V  L  L  F  V  A  V  H  G  A  V  G
caagccgtatgatattggtgtctcataaaggtcttctgtccccttgggaggtggcccagtttggatattagtaaaaattctcataagga 180

gcagtgctgcagtggcacagctgcccaggggggcggtggggtcaccatccctggaggtgttcaaatgtggagatgtggcgctgagggaca 270

tagtgggcagtgggcacggtgggggtggggttggacttggggatcttataggtcttttccaacctgagtgattctatgattccatgaata 360

gggtggtaagtgtcctccaggtgattatggatgggaaaagactgtgacggattgagaaagagaagggagaagtgggagaaatatcgtatc 450

tgcaacagtctccctttttctttttctttttttttcaatttttctttttctctttttttaaatactgcagGCTTTTCCCGTTCTCCAAGAT 540
                                                                        F  S  R  S  P  R  Y
ATCACATGCAATGTGGATATCGCGGGACCTTCTGCACCCCTGGGAAATGCCCTCATGGGAATGCTTACCTGGGGCTATGCCGTCCCAAGT 630
  H  M  Q  C  G  Y  R  G  T  F  C  T  P  G  K  C  P  Y  G  N  A  Y  L  G  L  C  R  P  K  Y
ATTCTTGCTGTAGATGgtaagattaagacttgactatggctaaactgacttcccagattttaagttctatatggtgggatttccccttc 720
  S  C  C  R  W
aacttaggtgtgaaaaccctgtactcttctttctttgcatagGTTGTAG 770
                                           L  *
```

AvBD5

```
ATGCAGATCCTGACTCTCCTCTTTGCTGTCCTCCTCCTGATGCTCCGGGCAGAACCAGgtgagatatacatacgttgtgggagggtggtg 90
M  Q  I  L  P  L  L  F  A  V  L  L  L  M  L  R  A  E  P  G
tgtttgccctttgttgatattttgtaggggataatggagggtttgatgatgattggtcatagaatcatagaatggcctgggttgaatgat 180

catccagtttcaaccccccctgctatgtgaagggtcaccaaccagcagaccaggctgcccagagccacatccagcctggccttgaatgcct 270

ccagggatggggcatccacttactgtggtacccaatggatttccattggaaagtttgccttggctggtagaaaaaaaaggaagaataggca 360

gcccaggggtgtggggagagctttccacttgtgttcagcaaggagacagtcagggtgcaccgatgttggctgtacaggggcagaaggctg 450

cgctcacagctgggcagaactgtgctgaggtgttctccttctgctctctgcagGGCTGTCCCTTGCTCGAGGATTACCCCAGGACTGTGA 540
                                                       L  S  L  A  R  G  L  P  Q  D  C  E
GCGCCGTGGGGGCTTCTGCTCCCACAAGTCATGTCCTCCAGGGATCGGCCGCATTGGCCTCTGCTCCAAGGAAGACTTCTGCTGCCGGAG 630
 R  R  G  G  F  C  S  H  K  S  C  P  P  G  I  G  R  I  G  L  C  S  K  E  D  F  C  C  R  S
gtaggctcagcgctgcctgatgcggggtggctgcttcctgttggggttgggggtgaggtccttgaagaagggaaataacacacagcccaa 720

tggcatgggggcatccccgggtccctgctactgcgttatccaaactgggagatgctgctggggctgcagcaatccggtgtcctccttcca 810

ccactaatgttggcagcccagccaccacctgtagagagcatggggactcttctcaggcttccaccagccccagaaccgtagttcagaagc 900

agtcccaaagggagatgggcattttaactgagggttctggcctcatagagttgggatgaacactgccacacctttcctcccacagCCGAT 990
                                                                                        R  W
GGTATTCCTGA 1001
  Y  S  *
```

to code for gallinacin 1α. Interestingly, the chicken genome sequence codes for AvBD1 (Figure 3.2). As well as the three nucleotide differences in the coding sequences, there were also nine nucleotide differences in the introns, seven in the first intron and two in the second intron. There is only one gene in the chicken genome with the potential to encode AvBD1 or gallinacin 1α and I propose that the two sequences represent polymorphic variants of the same gene.

3.2.3 A novel chicken β-defensin, AvBD14

The cDNA of the novel AvBD was first identified by Dr N. Bumstead and its gene sequence was then determined after using specific primers (Table 2.3) to amplify the gene from genomic DNA of line N and 6_1 chickens. After optimization of the PCR, the annealing temperature required to amplify the genomic DNA of AvBD14 was 55°C. The genomic sequence obtained showed that the AvBD14 gene was composed of two exons and one intron (Figure 3.3).

The thirteen AvBDs identified to date have been localised to chicken chromosome 3 and they appear to be the result of gene duplication events. Indeed, AvBDs 6 and 7 share the same signal peptide and have high similarity in their coding sequence as well as their intronic sequences (Figure 3.4). AvBD genes are generally composed of three exons and two introns, except those for AvBDs 11, 12 and 13, which have two exons and one intron (Figure 3.5). The AvBD14 genomic DNA sequence (Accession no. AM402953) has been recently identified in the current version of the genome sequence, part of its sequence being localised in contig17.130, on chromosome 3 at one end of the avian beta-defensin locus. An alignment of the relevant sequences showed only three nucleotide differences in the intronic sequence in 402 nucleotides (Figure 3.6). However, AvBDs gene alignments did not allow the identification of promoter regions of chicken defensins (see Appendix 6).

Figure 3.2: Comparison of the gene sequences and predicted amino acid sequences of AvBD1/1α from line 6_1 and N chickens (N/6) and the red jungle fowl, the source of the chicken genome sequence (genome). For the gene sequences, exons are in upper case, introns in lower case. Differences between the two sequences are highlighted.

```
N/6      ATGCGGATCGTGTACCTGCTCCTCCCCTTCATCCTCCTCCTGGCCCAGGGTGCTGCAGgtgaggtgtgagttctgtggggttctccatat 90
Genome   ATGCGGATCGTGTACCTGCTCCTCCCCTTCATCCTCCTCCTGGCCCAGGGTGCTGCAGgtgaggtgtgagttctgtggggttctccatat 90
N/6      M  R  I  V  Y  L  L  L  P  F  I  L  L  L  A  Q  G  A  A  G
Genome   M  R  I  V  Y  L  L  L  P  F  I  L  L  L  A  Q  G  A  A  G

N/6      cccaggaggtggcttgtcagggatgggtaacgactaggagggctctgatcagttggttcaggagggagggaagatttaggttggatatca 180
Genome   cccaggaggtggcttgtcagggatgggtaacgactaggagggctctgatcagttggttcaggagggagggaagatttaggttggatatca 180

N/6      gggggaagttctttacagagagagaggtgaggtgctggaacagctgcccagagaggctgtggatgccccgtccatccctggaggtgttca 270
Genome   gggggaagttctttacagagagagaggtgaggtgctggaacagctgcccagagaggctgtggatgccccgtccatccctggaggtgttca 270

N/6      aggccaggttggatggggccctgggcagcctgggctggtgttagatgtggaggttggtggccctgcctgtggtgggtgggttggagcttc 360
Genome   aggccaggttggatggggccctgggcagcctgggctggtattaaatggggaggttggtggccctgcctgtggtgggtgggttggagcttc 360

N/6      atgatccttgaggtcccttccaacccaaccattctgtgattctgtggtttggatgagtggctgggcttttgggtttggtgctttgtgcac 450
Genome   atgatccttggggtcccttccaacccaaccattctgtgattctgtggtttggatgagtggctgggcttttgggtttggtgctttgtgcgc 450

N/6      gtgttagactgagatccatgggacagccactctagaaccacacacagcttgtacaggtatcccacactcattttcttttggtctgtgcag 540
Genome   gtgttagactgagatccatgggacagccactctagaaccacacacagcttttacaggtatcctacactcattttcttttggtctgtgcag 540

N/6      GATCCTCCCAGGCTCTAGGAAGGAAGTCAGATTGTTTTCGAAAGAATGGCTTCTGTGCATTTCTGAAGTGCCCTTACCTCACTCTCATCA 630
Genome   GATCCTCCCAGGCTCTAGGAAGGAAGTCAGATTGTTTTCGAAAGAGTGGCTTCTGTGCATTTCTGAAGTGCCCTTCCCTCACTCTCATCA 630
N/6        S  S  Q  A  L  G  R  K  S  D  C  F  R  K  N  G  F  C  A  F  L  K  C  P  Y  L  T  L  I  S
Genome     S  S  Q  A  L  G  R  K  S  D  C  F  R  K  S  G  F  C  A  F  L  K  C  P  S  L  T  L  I  S

N/6      GTGGGAAATGCTCAAGATTTCACCTCTGCTGCAAAAGgtaagctttggaattagggatgaaattggatctgctaccacgatggcagaaat 720
Genome   GTGGGAAATGCTCAAGATTTTACCTCTGCTGCAAAAGgtaagctttggaattagggatgaaattggatctgctaccacgatggcagaaat 720
N/6        G  K  C  S  R  F  H  L  C  C  K  R
Genome     G  K  C  S  R  F  Y  L  C  C  K  R

N/6      agctgttgttgtgtttgatccccaaacctagctactggctttgggctatatatgatccagggcaggggcttggggaggaaaggagaaggt 810
Genome   agctgttgttgtgtttgatccccaaacctagctactggctttgggctatatatgatccagggcaggggcttggggaggaaaggagaaggt 810

N/6      gctaggaccggtcctttaaaggaactggaggaaccccagatcagacactggcctccccattgccctcagttacacggggctgcctggctt 900
Genome   gctaggaccggtcctttaaaggaactggaggaaccccagatcagacgctggcctccccattgccctcagttacacggggctgcctggctt 900

N/6      gctggtttcacaaatgcttccccagttggtgcagagtggagactctcccctgggtagtgtgaggcacagaacccattccctgatgtctct 990
Genome   gctggtttcacaaatgcttccccagttggtgcagagtggagactctcccctgggtagtgtgaggcacagaacccattccctgatgtctct 990

N/6      gcaaaaccttggaaaccaagctgaaaccaagctgtctgctatgcaggctgcttactacctgcattgagattagtgtcaatgtgtcagtgt 1080
Genome   gcaaaaccttggaaaccaagctgaaaccaagctgtctgctatgcaggctgcttactacctgcattgagattagtgtcaatgtgtcagtgt 1080

N/6      tatccaggagaagtgatgcatagtgagagacagaaaaaggagaataaaaagaggtgacctcacagagtgttttcttcctgcagAATATGG 1170
Genome   tatccaggagaagtgatgcatactgagagacagaaaaaggagaataaaaagaggtgacctcacagagtgttttcttcctgcagAATATGG 1170
N/6                                                                                          I  W
Genome                                                                                       I  W

N/6      GGCTGA 1176
Genome   GGCTGA 1176
N/6      G  *
Genome   G  *
```

Figure 3.3: Gene sequence and predicted peptide sequence of AvBD14. For the gene sequences, exons are in upper case and intron in the lower case.

```
ATGGGCATATTCCTCCTGTTTCTTGTTCTCCTGGCAGTACCCCAGGCTGCACCAGgtaag 60
M  G  I  F  L  L  F  L  V  L  L  A  V  P  Q  A  A  P  E
cgtaaatataatcaaaggtcatttttatgtttgggaaacagggaactgttctgcaaatga 120

agaatgaactcatggcacattgacgtgatgcttggtgctggatctggtagacgagtgttg 180

agtaggcaaactctggttggcactttcagggctggaggggagaacctcacaatctgcaac 240

tcatgaatgctttcagacatggcagcaaaactcaacagtgggttcacgttcctcttgctg 300

aactgacctgtgctacctgcaggctgagctggtagcatggagaccagccttcttcacact 360

tggaaatccaatggaagagtctcacaggttctttttctccattacagAGTCGGACACTGT 420
                                                S  D  T  V
CACATGTCGGAAGATGAAGGGCAAGTGTTCGTTCTTGCTGTGTCCTTTCTTCAAGAGATC 480
 T  C  R  K  M  K  G  K  C  S  F  L  L  C  P  F  F  K  R  S
CAGTGGTACCTGCTACAATGGACTGGCAAAGTGCTGCAGACCCTTTTGGTGA         532
 S  G  T  C  Y  N  G  L  A  K  C  C  R  P  F  W  *
```

Figure 3.4: Comparison of the genome sequences (Gal6/ Gal7) and amino acid sequences (G6 aa/G7 aa) of AvBD6 and 7. For the gene sequences, exons are in upper case and introns in lower case. The similarities between sequences are highlighted.

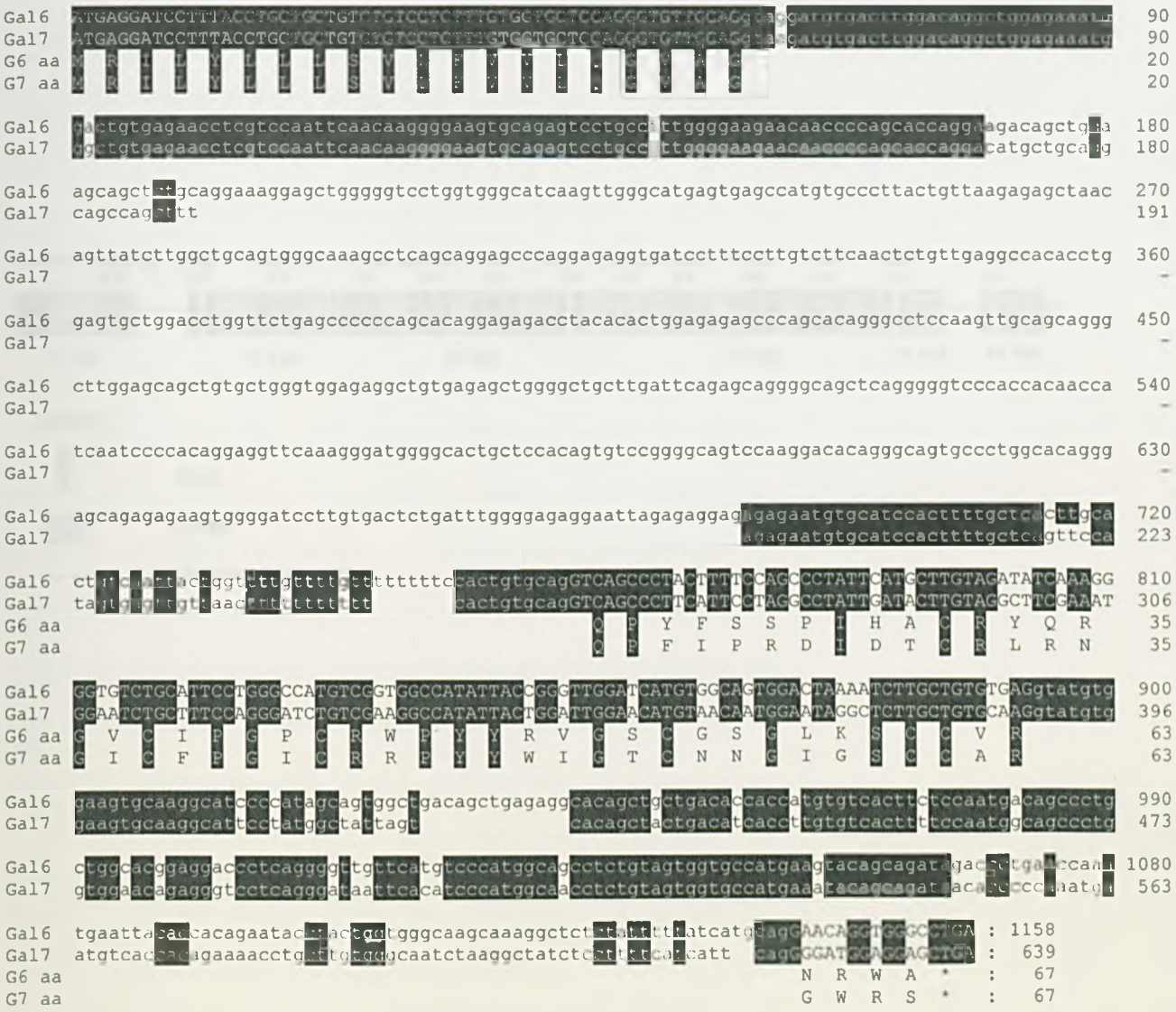


Figure 3.5: Genomic organisation of the AvBDs on chicken Chromosome 3 (adapted from Higgs *et al.*, 2005)

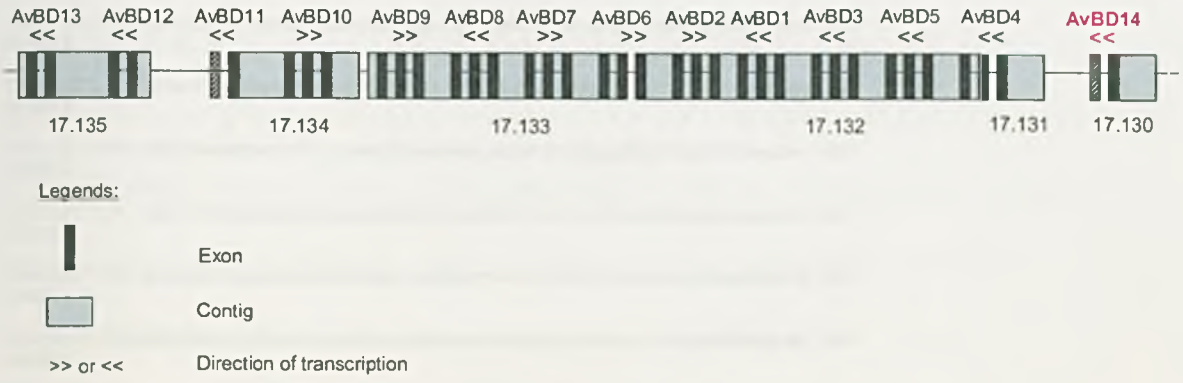


Figure 3.6: Comparison of the AvBD14 partial gene sequence from line 6_1 and N (AvBD14) and the red jungle fowl (the chicken genome) (Contig17130). For the gene sequences, exons are in upper case, introns are in lower case. Differences between the two sequences are highlighted.

```
Contig17130 ggtggattccccaccccactgcatcccatcccatcccatcccatcccatcccatccctta  60
AvBD14

Contig17130 aaatcccatcccatcccatcccaacccatatgagtgtttttgggggctttaactcatttt 120
AvBD14

Contig17130 tctgctgtttttttgtttgtttgtttgtttttaatctttagcaagtttcttggcaaccctt 180
AvBD14

Contig17130 ggaactaagcctggacactgctggtttaagcagcagtctgtcatccaactcggtttccag 240
AvBD14

Contig17130 agttctctatagatccccagtgtgtgatgcctgtctctctgtgccctatgcttccctctt 300
AvBD14

Contig17130 gatacttgcatatgaacagtgacccaaacctgtgtgctcagggatgtgctgctggctggg 360
AvBD14

Contig17130 gagccaagctactctctctgattttagaaggagtgtatctgggatctctgtctccagtac 420
AvBD14

Contig17130 tgatttttaccaactatggaagaactttgcatccttcatccttacatttaagcagcccct 480
AvBD14

Contig17130 gaatgaaaaaaggggtgcatggcctacagcctatttttcctcttggtgtgctaaatagcc 540
AvBD14

Contig17130 attcacatctccagtgaagcaaaatatagacagtggtgaaatcacctctgcacatggcat 600
AvBD14

Contig17130 ggcatgggctggcacaggatggcatagtggcatggggtgtctgccccatttttgcaggct 660
AvBD14

Contig17130 tattgaggtggggcatgttgtgctatgcatccacacaggaattgtaattagaggttacaa 720
AvBD14

Contig17130 gacacgtccttcaaagctgttatttataagattgctaaatccctggtgatcacattcatc 780
AvBD14

Contig17130 aaagctttataaagagaggctcattccttcctcttggtctcagcagcttcagggcgacac 840
AvBD14

Contig17130 gacaatgtcaaccaaagccATGGGCATATTCCTCCTGTTTCTTGTTCTCCTGGCAGTACC 900
AvBD14                          ATGGGCATATTCCTCCTGTTTCTTGTTCTCCTGGCAGTACC 41

Contig17130 CCAGGCTGCACCAGgtaagcgtaaatataatcaaaggtcatttttatgtttgggaaacag 960
AvBD14       CCAGGCTGCACCAGgtaagtgtaaatataatcaaaggtcatttttatgtttgggaaacag 101

Contig17130 ggaactgttctgcaaatgaagaatgaactcatggcacattgacgtgatgcttggtgctgg 1020
AvBD14       ggaactgctctgcaaatgaagaatgaactcatggcacattgacgtgatgcttggtgctgg 161

Contig17130 atctggtagacgagtgttgagtaggcaaactctggttggcactttcagggctggagggga 1080
AvBD14       atctggtagacgagtgttgagtaggcaaactctggttggcactttcagggctggagggga 221

Contig17130 gaacctcacaatctgcaactcatgaatgctttcagacatggcagcaaaactcaacagtgg 1140
AvBD14       gaacctcacaatctgcaactcatgaatgctttcagacatggcagcaaaactcaacagtgg 281

Contig17130 gttcacgttcctcttgctgaactgacctgtgctacctgcaggctgagctggtagcatgga 1200
AvBD14       gttcacgttcctcttgctgaactgacctgtgctacctgcaggctgagctggtagcatgga 341

Contig17130 gaccagccttcttcacacttggaaatccaatggaagagtctcacaggttctttttctcca 1260
AvBD14       gaccagccttcttcacacttggaaatccaatggaagagtctcacaggttcattttctcca 401

Contig17130 t 1261
AvBD14       ttacagAGTCGGACACTGTCACATGTCGGAAGATGAAGGGCAAGTGTTCGTTCTTGCTGT 461

Contig17130
AvBD14       GTCCTTTCTTCAAGAGATCCAGTGGTACCTGCTACAATGGACTGGCAAAGTGCTGCAGAC 521

Contig17130
AvBD14       CCTTTTGGTGA 532
```

3.2.4 Characterisation and cloning of AvBD cDNAs

The cDNA of HD11 macrophage-like cells and tissues such as the spleen, lung, bursa of Fabricius and bone marrow from chicken lines N, 6_1 and 7_2 at 1, 6 and 7 weeks of age were used to obtain defensin cDNAs. The primers used to obtain these cDNAs are listed in Table 2.3. AvBD1 (Gal 1α form) and AvBD2 cDNAs were obtained from lung tissue of 1 week old line N chickens, with an annealing temperature of 58°C. AvBD3 cDNA was obtained from spleen tissue of 7 week old line N chickens, with an annealing temperature of 85°C. AvBD4 and AvBD14 were obtained from a pool of spleen cDNA (provided by Prof John Young, IAH), with an annealing temperature of 55°C. Finally, AvBD5 cDNA was obtained from HD11 macrophage-like cells cDNA with an annealing temperature of 58°C (Figure 3.7). The additional bands obtained were probably unspecific amplification of genomic DNA as the RNA samples extracted from tissues were not treated with DNase. In order to express AvBD mRNAs and AvBD1 (Gal 1α form), 2, 3 and 4 peptides, appropriate restriction sites were inserted by PCR to ligate the AvBD cDNAs into the *Nco*I restriction site of the pTriEx.1.1 expression vector (Figure 3.8). After cloning into pTriEx 1.1, the cDNA sequences were checked by sequencing and either used to produce AvBD peptides in a Baculovirus system, or to express AvBD mRNAs in COS-7 cells for Taqman standards.

3.2.5 Evolutionary analysis of chicken β-defensins

The vertebrate defensins are classified in three subfamilies, α-, β- and θ-defensins, with only β-defensins identified in chickens. A phylogenetic tree with other vertebrate β-defensins, such as human, bovine and mouse, shows that individual AvBDs cluster with different groups of mammalian defensins (Figure 3.9). β-defensins are a major subfamilies, which arose before the divergence of birds and

Figure 3.7: Electrophoresis gel of defensin cDNAs. Arrows indicate the AvBDs cDNA sequence that have been purified; AvBD1 (198 bp); AvBD2 (195 bp); AvBD3 (243 bp); AvBD4 (185 bp); AvBD5 (189 bp); AvBD14 (180 bp); Ma, Marker.

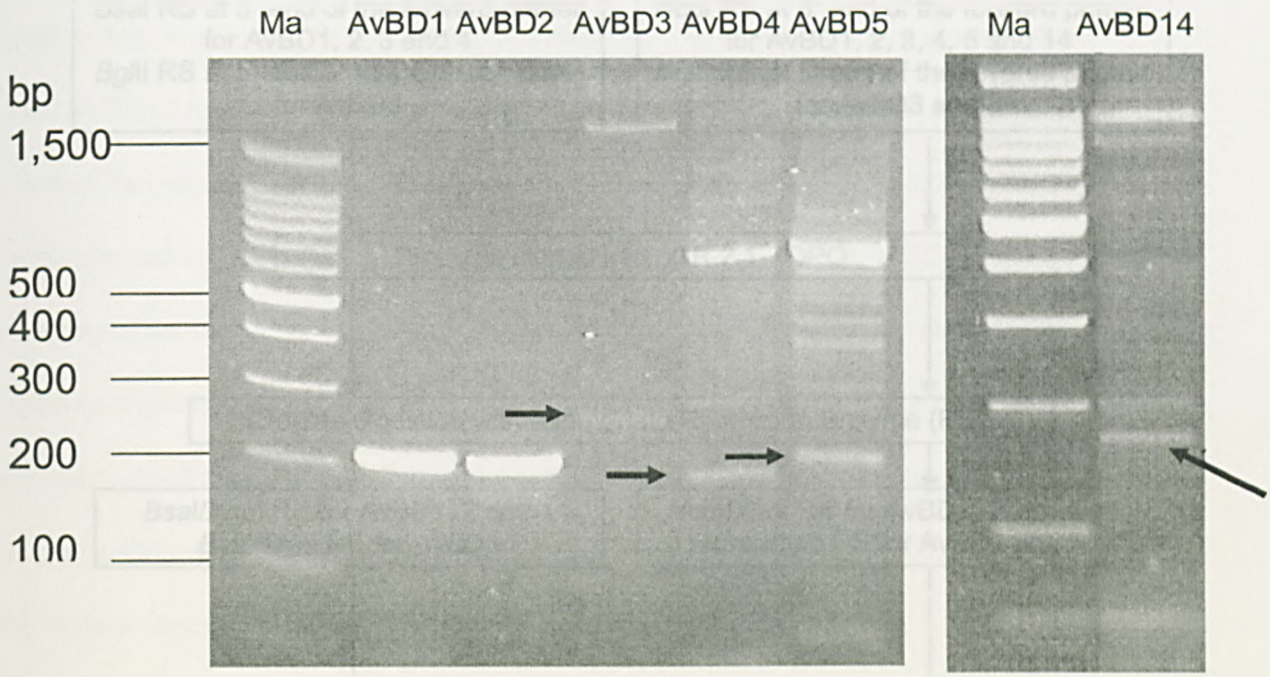
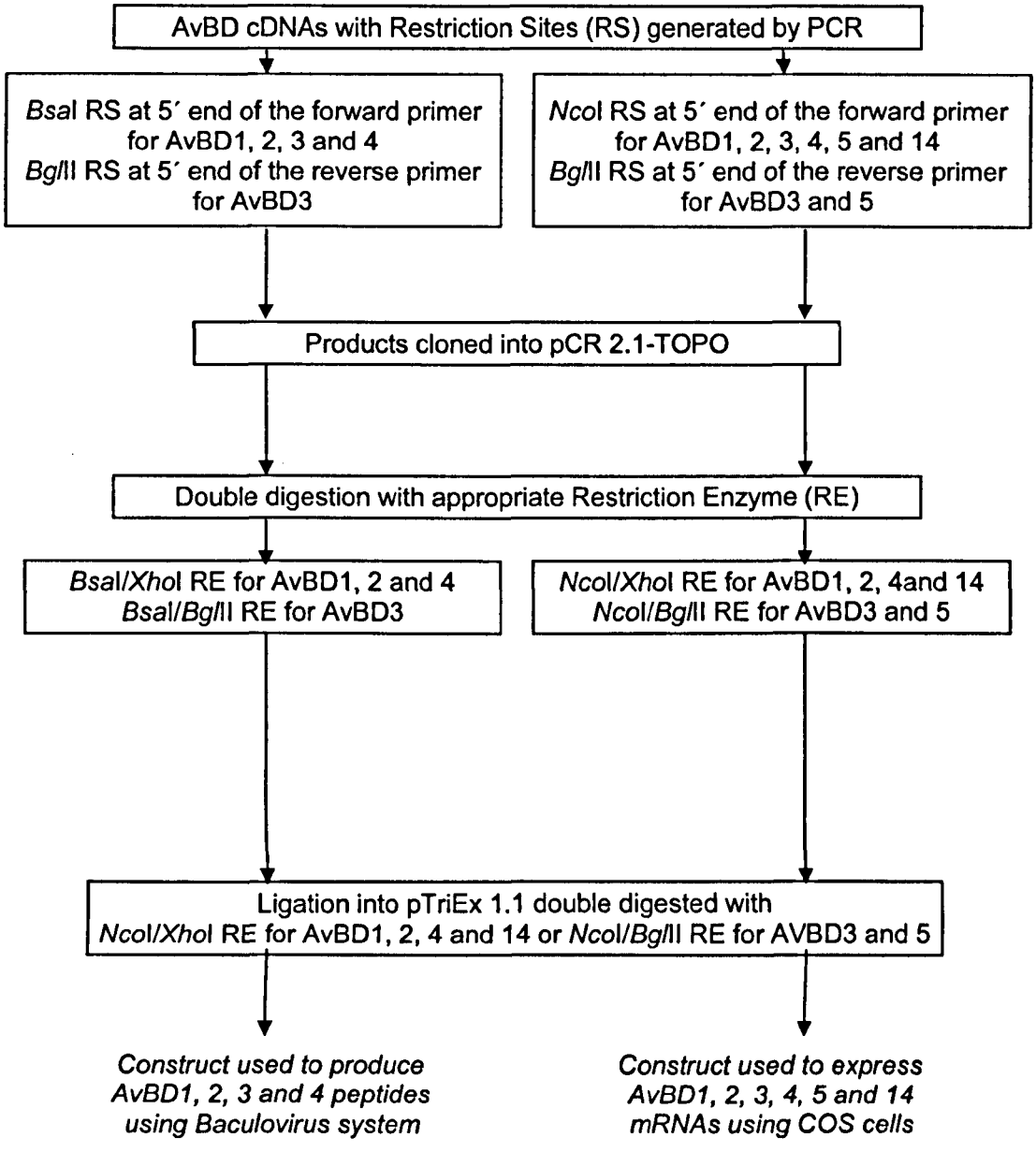


Figure 3.8: Schematic diagram showing the cloning strategy for the AvBD cDNAs into pTriEx 1.1 expression vector.



mammals from a common ancestor. The α-defensins, identified only in mammals so far, may have evolved after this divergence. The release of the chicken genome allowed the identification of ten more sequences coding for β-defensins (Figure 3.10). The nomenclature used is based on Lynn *et al.* (2007). AvBD11 is the sequence described by Xiao *et al.* (2004) only and AvBD14 is the novel sequence described here. AvBD13 has two different sequence predictions. The sequence underlined with a dotted line was predicted by Xiao *et al.* (2004), while the Higgs *et al.* (2005) prediction did not contain this sequence. The main characteristic of AvBDs is the presence of six cysteines that form three pairs of disulphide bridges. In addition, the tripartite sequence common to other β-defensins was also identified with a signal peptide, a small propiece and finally the mature peptide, that contains specific features such as a short sequence, a cationic net charge, a lack of glycosyl modification and the tertiary structure of a β-sheet dimer.

3.3 Discussion

Defensins are antimicrobial peptides and are an important component in mucosal host defences to prevent the invasion of enteric pathogens (Wilson *et al.*, 1999). The vertebrate defensins are classified in three subfamilies, α-, β- and θ-defensins, that could derive from a common evolutionary origin. Indeed, only β-defensins have been identified in the two "old" classes of vertebrate, reptile and avian (Sugiarto & Yu, 2004), and the clustering of AvBDs, the chicken's β-defensins, with mammalian β-defensins, as observed in phylogenetic trees, suggested that this might be the original defensin family (Xiao *et al.*, 2004). In addition, comparative analysis revealed that chicken β-defensin gene cluster is syntenic with two clusters on human 8p22 and 8p23.1 and their orthologous loci in other mammalian species including rat,

Figure 3.9: Phylogenetic analysis of vertebrate β-defensins. Phylogenetic tree generated with Phylip software. MBD, mouse β-defensin; HBD, human β-defensin; BNBD, bovine neutrophil β-defensins; LAP, lingual antimicrobial peptide; EBD, enteric β-defensin and AvBD, avian β-defensin. HBD1, NP_005209; HBD2, AF040153; HBD3, AF295370; HBD4, AJ314835; MBD1, AH005574; MBD2, AJ011800; MBD3, AF093245; MBD4, AF155882; MBD5, AF318068; MBD6, AB063109; MBD27, AY591384; MBD30, DQ141309; MBD36, AY591385; BNBD3, AF016396; BNBD4, AF014107; LAP, NM203435; EBD, AF016539; LAP, NM203435; AvBDs accession numbers are listed in Table 1.4.

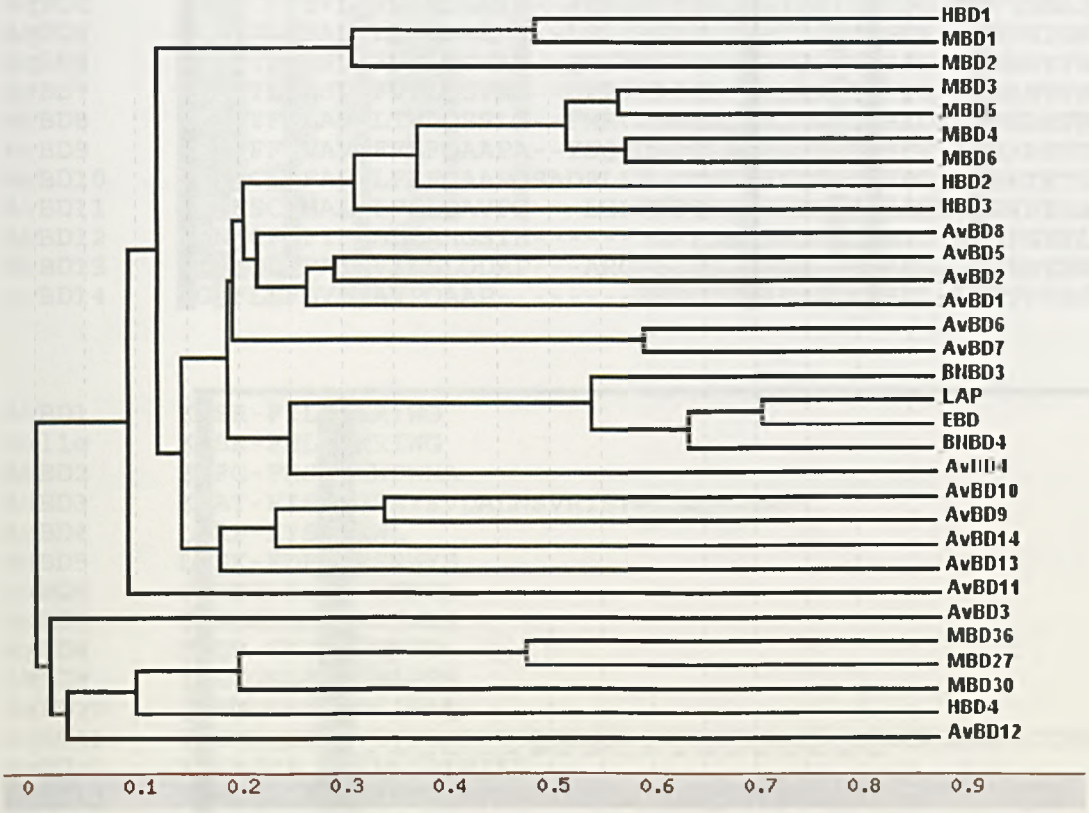
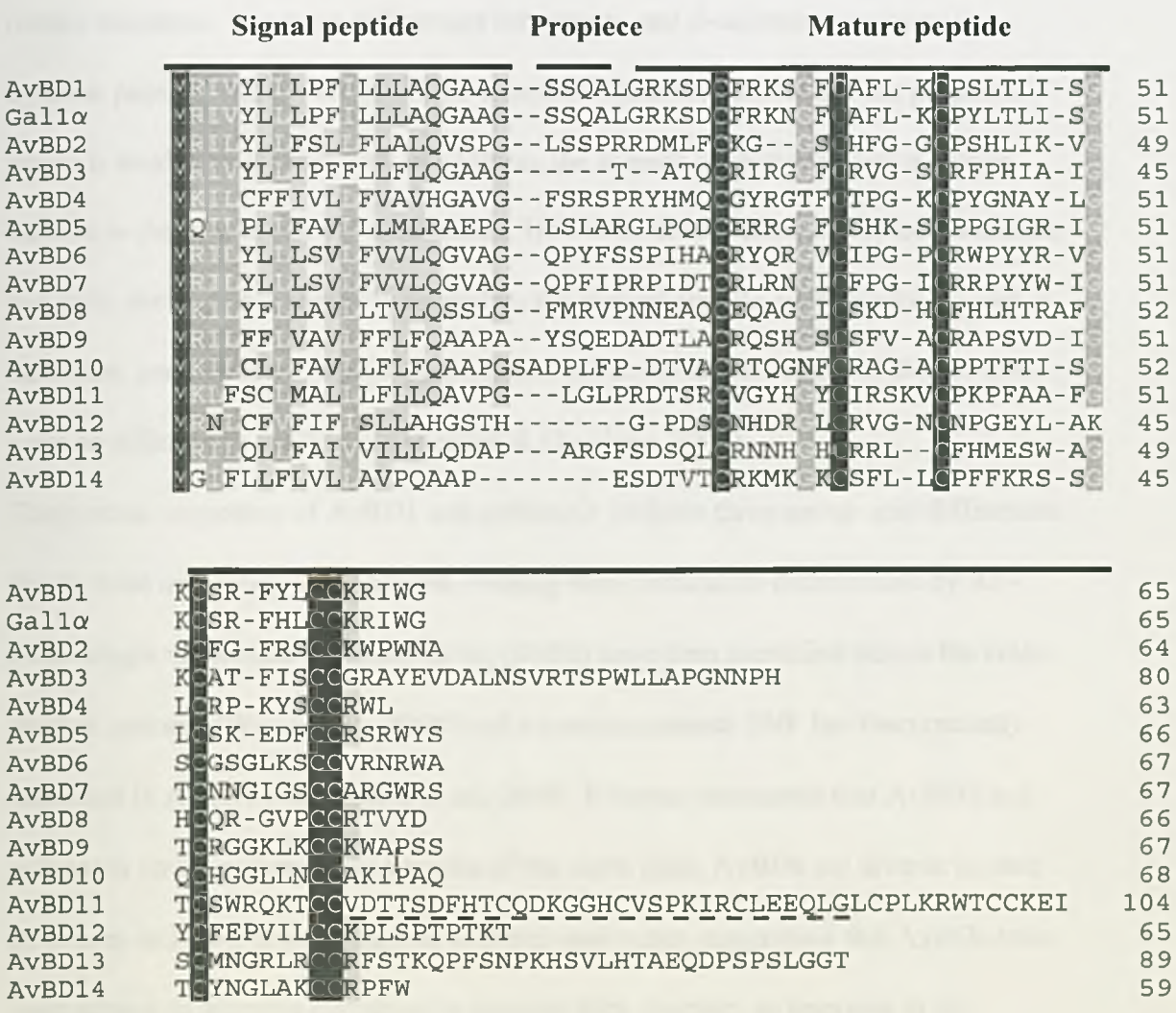


Figure 3.10: Amino acids pile-up of the avian β-defensins. The multiple sequence alignment was generated with Clustal X (1.83). The dotted line represents the part of AvBD13 sequence predicted by Xiao *et al.* (2004) but absent from Higgs *et al.* (2005) prediction.



mouse and dog (Patil *et al.*, 2005) suggested that all vertebrate β-defensins are evolved from a single gene.

The prepropeptide of defensins is composed of a signal sequence, a propiece and a mature sequence. The main differences between α- and β-defensins concerns the cysteine pairings and the length of the structural features, particularly the propiece, which is smaller in β-defensins. In addition, the anionic propiece confers a charge balance to the propeptide for α-defensins. However, the β-defensin propiece is shorter and lacks the negative charge to neutralize the mature peptide, particularly chicken β-defensins, and therefore the biosynthesis and intracellular trafficking of β-defensins must be different to α-defensins (Selsted & Ouellette, 2005).

The peptide sequences of AvBD1 and gallinacin 1α have three amino acid differences due to three nucleotide substitutions, making them difficult to differentiate by RT-PCR. Single nucleotide polymorphisms (SNPs) have been identified across the entire chicken genome (Wong *et al.*, 2004) and a nonsynonymous SNP has been recently identified in AvBD5 (Hasenstein *et al.*, 2006). It seems reasonable that AvBD1 and gallinacin 1α are polymorphic variants of the same gene. AvBDs are diverse in their structures and their potency against different pathogens, suggesting that AvBDs have been subject to adaptive evolution to increase their diversity in response to the constant increase in diversity of microbial pathogens. Therefore, the sequence differences between AvBD1 and gallinacin 1α may be explained by adaptive evolution. However, it would be interesting to compare the activity and efficiency of the two different forms against different pathogens.

Following the release of the chicken genome sequence (version 2.1- August 2006) (Wong *et al.*, 2004), ten AvBD sequences were identified via bioinformatics. My novel avian β-defensin, AvBD14, remains only partially characterised in the genome.

AvBD14 was first identified in BAC bW094K17 from the the Wageningen library in this laboratory, which was subsequently sequenced at the Beijing Genomics Institute. BAC bW094K17 Contig14 also encoded for two new AvBDs, published since then as AvBDs 4 and 5, and also the published AvBDs 1 and 3. Therefore, the BAC bW094K17 Contig14 identified in this laboratory in July 2002 corresponds to Contigs 17.130, 17.131 and the beginning of 17.132 in the chicken genome to date (Figure 3.5). The determination of the novel AvBD14 genomic sequence by PCR indicated a gene organisation of two exons and one intron, as is the case for AvBDs 11, 12 and 13, the other AvBD genes all having 3 exons and 2 introns. However, this gene structure could be incorrect as the full gene sequence of AvBD14 is absent in the chicken genome sequence and the third exon of AvBD genes tends to contain only a few nucleotides, which cannot be identified by bioinformatics approaches alone.

In summary, genomic DNA sequences for AvBDs 1, 2, 3, 4, 5 and 14 have been characterised. The corresponding cDNAs have been isolated and successfully cloned in the pTriEx1.1 expression vector for expression in COS-7 cells to generate AvBD mRNA standards for Taqman analysis. In addition, AvBDs 1, 2, 3 and 7 have been cloned correctly into pTriEx1.1 to express these AMPs in the Baculovirus system.

Chapter 4 - Expression of AvBDs 1, 2, 3 and 4

4.1 Introduction

Defensins have been identified in all mammals so far studied, as well as poultry, insects, invertebrates and plants (Ganz, 2003b). They are synthesised in granulocyte cells or secreted by epithelial cells and contribute to host defence against microbial colonisation and infection.

Defensins are expressed either constitutively or in response to infection. In mammals, the α-defensins are generally synthesised and stored in granules in mature leukocytes. They are also synthesised by Paneth cells in an inactivate form (Raj & Dentino, 2002). Immature defensins consist of a tripartite prepropeptide with a precursor sequence of 90-100 amino acids containing an amino (N)-terminal signal sequence of about 19 amino acids, an anionic propiece of about 45 amino acids and a carboxy (C)-terminal mature cationic defensin of about 30 amino acids. The negative charge of the propiece usually neutralises the positive charge of the mature defensin preventing the premature interaction of defensins with the membranes of neutrophils and Paneth cells. The process of maturation involves enzymes such as the metalloproteinase, matrilysin, in mice (Wilson *et al.*, 1999) or three forms of trypsin in human (Ganz, 2003b). Conversely, β-defensins are synthetised by epithelial tissues only in mammals and the "prepro-β-defensins" have a very short propiece suggesting that intracellular transportation of α- and β-defensins is different.

The avian β-defensins stored in heterophil granules consist also of tripartite prepropeptide sequences with a precursor sequence containing an N-terminal signal sequence, a basic or neutral propiece and a mature cationic defensin of about 40 amino acids. However, the avian β-defensin propeptide is unable to neutralise the mature peptide. AvBD1 is not negatively charged whereas AvBD2 has only one

negative charge. Therefore, some other mechanism must be involved in neutralising premature activity in these β-defensins (Brockus *et al.*, 1998).

The post-translational modification of defensins is important to consider when producing AvBDs. Different methods have been used to produce human β-defensins. Despite their antimicrobial activity, their production was attempted in an *E. coli* expression system (Piers *et al.*, 1993). However there are only a few descriptions of using this system successfully to produce defensins and the correct folding of proteins with a high numbers of cysteines is difficult to obtain using bacteria as the expression host (Harder *et al.*, 2001). Therefore, despite success in expressing human β-defensin 3 and human α-defensin 1 in *E. coli*, this method has not been used to produce AvBDs. Similarly COS-7 cells, *Pichia pastoris* and *Bacillus subtilis* have been used to produce human defensins, but all gave low levels of expression only detectable by Western blotting (Chen et al., 2006). Recently, human defensins tend to be produced synthetically (Yang *et al.*, 2004). In addition to its efficiency, this system allows the production of the mature peptide only and should create the proper disulphide bridges for correct folding of the defensin peptide. Finally, recombinant baculovirus-infected insect cells have been used to produce human defensins. Both human α- and β-defensins have been expressed with success (Liu *et al.*, 2002; Valore *et al.*, 1998) and the system expressed the mature form of the peptide directly into the supernatant (Bals *et al.*, 1998). Based on these observations, I chose this expression system to produce AvBDs using a bacmid (Bac10:KO_{1629}) baculovirus DNA, improved by Professor Ian M. Jones at the University of Reading (Zhao *et al.*, 2003), which was previously used with success to express the envelope glycoprotein E2 of bovine viral diarrhoea virus (BVDV) (Pande *et al.*, 2005).

This Chapter presents the strategy used to produce AvBDs 1 (Gal1α form), 2, 3 and 4 peptides (Figure 4.1). Because of their low molecular weight and the importance of obtaining correct folding to properly test their activity, no tags were added to the 3' end of the AvBD cDNAs as previously described (Gueguen *et al.*, 2006; Satchell *et al.*, 2003; van Dijk *et al.*, 2007). In addition, the absence of monoclonal antibodies against AvBDs necessitated the choice of a multi-step purification method to obtain the AvBD peptides, including cationic exchange followed by Reverse Phase-High Performance Liquid Chromatography (RP-HPLC).

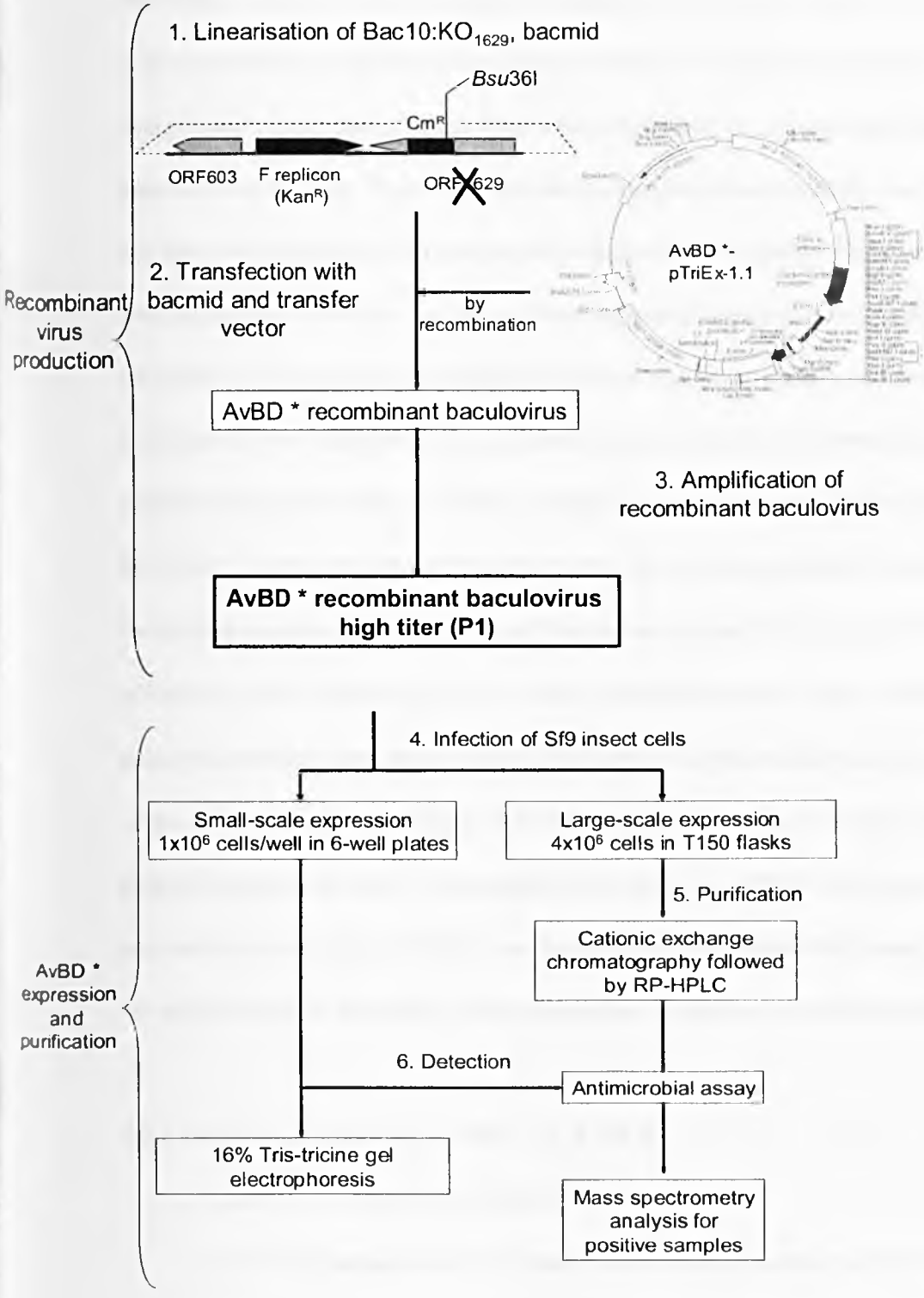
4.2 Methods

4.2.1 Expression of and purification strategy for AvBDs 1, 2, 3 and 4

AvBD cDNAs were cloned in pTriEx 1.1 transfer vectors into the *Nco*I restriction site downstream of a start codon to express the target gene. The pTriEx 1.1 vector contains flanking baculovirus sequences allowing the production of viable virus only if a recombination event occurs between the bacmid, Bac10:KO_{1629}, and the transfer vector. Therefore, transfection with the transfer vectors and the previously linearized bacmid yielded recombinant baculovirus coding for AvBDs.

A small-scale, followed by a large-scale, expression experiment was carried out at the University of Reading in Prof Ian Jones' laboratory. The small-scale expression would estimate the magnitude of scale-up required to produce the desired amount of protein, while the large-scale expression yieded protein for subsequent purification. The purification selected was a multi-step purification including cationic exchange chromatography, followed by RP-HPLC. Ion exchange chromatography is based on absorption and reversible binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix. The pH value at which a peptide is neutral,

Figure 4.1: Schematic diagram showing the production strategy for AvBDs peptides. The star (*) represents the different AvBD numbers.



carrying no net charge, is called the isoelectric point (pI). When exposed to a pH below its pI, the peptide will carry a positive charge and will bind to a cation exchanger, whereas when the peptide is exposed to a pH above its pI, it carries a negative charge and will bind to an anion exchanger. AvBDs are naturally positively charged and this characteristic is essential for their activity, particularly their antimicrobial activity. Therefore, cationic exchange chromatography was performed and the protein eluted with a continuous salt gradient at constant pH between 6 and 8. The elution step of the ion exchange chromatography can also be carried out by changing the pH, which will change the protein charges gradually and allow the collection of very sharp peaks in a minimal elution volume. However, this method could denature and inactivate AvBDs. Finally, the samples were further purified by RP-HPLC. In reverse phase chromatography, the stationary phase is non-polar and the mobile phase is moderately polar. The retention time is the result of the interaction of the non-polar components of the solutes with the stationary phase, allowing the polar molecules to elute more readily. This chromatography allows a good separation of the ionic proteins according to their hydrophobicity, which is roughly inversely proportional to solute size. The retention time increases with the hydrophobic surface area and so the addition of trifluoroacetic acid (TFA) to the mobile phase, acting as an ion pairing agent to neutralize molecule charges, improves the chromatography.

4.2.2 Detection strategy for AvBDs 1, 2, 3 and 4

4.2.2.1 Tris-Tricine SDS-PAGE

One of the methods used to identify the correct expression of the AvBD peptides was SDS-PAGE. A 16.5% Tris-Tricine gel, separating proteins with a molecular weight range of 4-30 kDa, was used to assess avian β-defensin production

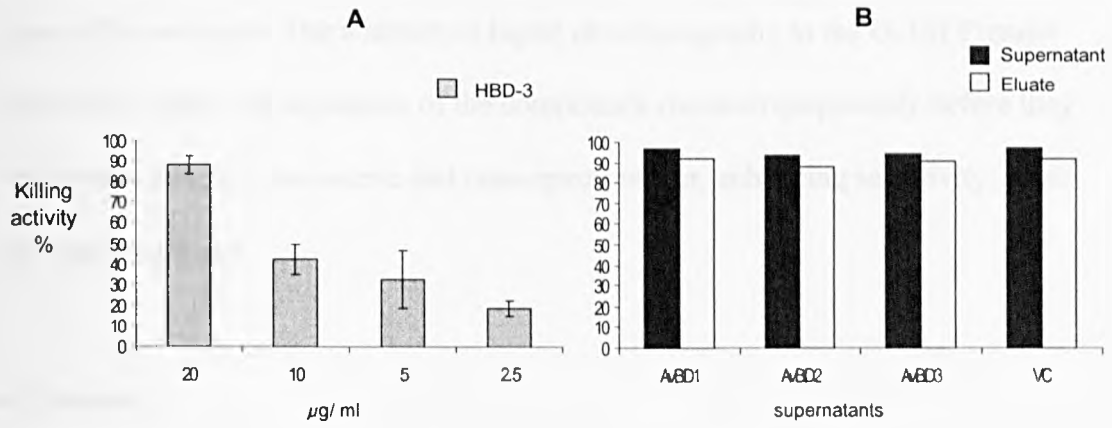
in supernatants after small-scale expression. In addition, the Tricine buffer system allowed the separation of small SDS-coated proteins from SDS micelles.

4.2.2.2 Antimicrobial assay

Defensins were first described as AMPs. Their antimicrobial activity is based on non-specific interaction of the positively charged peptide with the negatively charged bacterial membrane. This specific characteristic was therefore used to detect AvBDs throughout the multi-step purification process.

The antimicrobial assay was carried out with a defensin-sensitive strain, *E. coli* BUE55 and human β-defensin 3 (HBD3) as a positive control, provided by Dr D. Devine, University of Leeds. The optimisation of the assay was performed with HBD3 at different concentrations. The data showed (Figure 4.2A) that the best concentration to detect a significant killing activity repeatedly was 20 μg/ml and this concentration was therefore used in each assay with HBD3 as the positive control. Thereafter, the killing activity tended to decrease with decreasing HBD3 concentration (Figure 4.2A). The baculovirus system expressed around 3-5 μg/ml of human neutrophil peptide-1 (HNP-1) α-defensin, which could be detected by the antimicrobial assay despite the low percentage of killing activity obtained with these concentrations. The negative control used to determine the percentage of killing of HBD-3 was sterile distilled water, while the negative control used to determine the killing activity of samples was buffer or media, in which the samples to be tested were contained.

Figure 4.2: Antimicrobial assays. A. Antimicrobial activity of HBD-3 against *E. coli* BUE55. Negative control contained sterile distilled water only. Results are expressed as % ± S.E.M. (n=3). **B. Antimicrobial assay of supernatant and eluate to compare AvBD1, 2 and 3 activities with eluate of the virus control.** The supernatant was obtained after infection with AvBD1, 2, 3 or virus control (VC) recombinant baculoviruses and represent the medium obtained after centrifugation (e.g. **2.17 Expression of AvBDs in Baculovirus system**- small and large-scale production). The eluate was obtained after cationic exchange chromatography, CM Sepharose™ Fast Flow (e.g. 2.19.1 Cationic exchange chromatography). The negative control was distilled water. The full test was carried out only once.



4.2.2.3 Mass-spectrometry analysis

The detection of AvBDs expressed from insect cells was also carried out using a Q-Tof Premier (Waters) mass spectrometer to determine their presence and the purification quality according to their molecular weight. The Quadrupole/Time-of-flight (Q-Tof) instruments allow the identification of small molecules in a complex sample. Indeed, with the combination of the quadrupole, which acts as a mass selective filter, and the Tof, which detect the ions according to their charge and their velocity, the Q-Tof instruments demonstrate good selectivity and determine the exact mass of the molecule. The addition of liquid chromatography to the Q-Tof Premier instrument allows the separation of the compounds chromatographically before they are introduced to the ion source and mass spectrometer, enhancing sensitivity, mass accuracy and speed.

4.3 Results

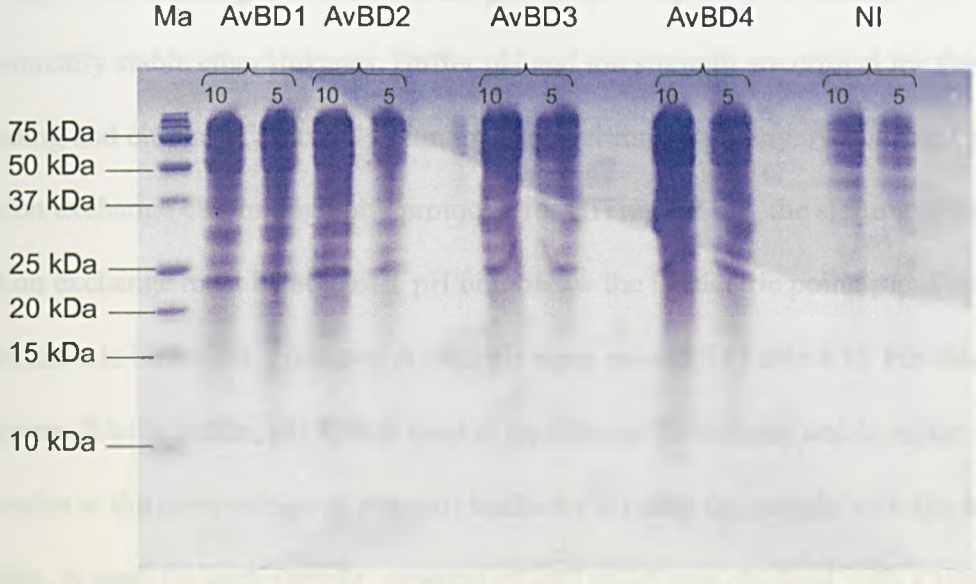
4.3.1 Small-scale expression of AvBDs 1, 2, 3 and 4

The predicted molecular weights of the AvBDs containing the prepropeptide or the mature peptide only were all less than 10 kDa (Table 4.1). However, the supernatants obtained after small-scale expression and run on a 16.5% Tris-tricine gel did not give any strong bands corresponding to the predicted avian β-defensin molecular weights (Figure 4.3). A similar gel was then silver-stained but it did not show any differences (data not shown). Bands higher than 10 kDa present in the test samples but not in the uninfected control might represent viral proteins. Based on previous publications, electrophoresis gels are usually used to check the purity of

Table 4.1: Avian β-defensin peptide sequences and their physico-chemical parameters. The mature peptide is in bold. MW, molecular weight; pI, isoelectric point.

	Amino acid sequence	Prepro-peptide		Mature peptide	
		MW (kDa)	pI	MW (kDa)	pI
AvBD1 (Gal1α)	MRIVYLLLPFILLLAQGAAGSSQAL**GRKSDCFRKNGFCAFL KCPYLTLISGKCSRFHLCCKRIWG**	7.3	9.8	4.7	9.7
AvBD2	MRILYLLFSLLFLALQVSPGLSSPR**RDMLFCKGGSCIIFGGC PSHLIKVGSCFGFRSCCKWPWNA**	7.1	9.4	4.3	8.9
AvBD3	MRIVYLLIPFFLLFLQGAAGTA**TQCRIRGGFCRVGSCRFPIII AIGKCATFISCCGRAYEVDALNSVRTSPWLLAPGNNPII**	8.5	9.4	6.4	9.2
AvBD4	MKILCFFIVLLFVAVHGAVGFSRSP**RYIHMQCGYRGTFCTPG KCPYGNAYLGLCRPKYSCCRWL**	7.2	9.5	4.5	9.3

Figure 4.3: Tris-Tricine SDS-PAGE of supernatants obtained after centrifugation of insect cells infected with AvBD recombinant viruses for 3 days (e.g. **2.17 Expression of AvBDs in Baculovirus system)** to detect AvBDs that can be secreted directly in the supernatant (Bals *et al.*, 1998). The gel was then Coomassie stained. 10 and 5 μl of supernatants were mixed with 5 and 2.5 μl of SDS-loading buffer respectively and the mixture was then loaded into the electrophoresis gel. Ma, marker; AvBD1, avian β-defensin 1; AvBD2, avian β-defensin 2; AvBD3, avian β-defensin 3; AvBD4, Avian β-defensin 4; NI, non-infected insect cells as control.



defensins after purification. Therefore, large-scale expression followed by purification was performed.

4.3.2 Large-scale expression and small-scale purification of AvBDs 1 and 2

Purification was carried out with both the supernatant and the pellet obtained from the large-scale expression of AvBD1. SP Sepharose High Performance is a strong cation exchanger. The functional groups are coupled to the matrix via chemically stable ether linkages. Buffer pH and ion strength are critical for the binding and elution of material in ion exchange chromatography. According to the cation exchange chromatography protocol, for HiTrap SP HP, the starting pH for cation exchange must be at least 1 pH unit below the isoelectric point, pI, of the substance to be bound. Predicted AvBD pIs were around 9 (Table 4.1). For this purpose, Bicine buffer, pH 8, was used to equilibrate the column and to adjust the samples to the composition of the start buffer by diluting the sample with the start buffer. A peak for each sample, supernatant and pellet was obtained with a slight difference in retention time (Figure 4.4). Indeed, the peak obtained from the pellet sample eluted later than that from the supernatant. The samples were then further purified by RP-HPLC and the different peaks obtained tested for their antimicrobial activity (Figure 4.5). The RP-HPLC pattern of the supernatant was different to the pellet. The pellet sample had two distinct peaks, while the supernatant sample had one distinct peak. The antimicrobial assay for the different samples showed killing activity against *E. coli* BUE55. The samples were, therefore, run on a 16.5 % Tris-Tricine gel. However, both Coomassie staining and silver staining did not reveal protein bands of the expected size (data not shown). Consequently, the positive samples were then analysed by mass spectrometry but none of them contained a peptide corresponding to

Figure 4.4: Cationic exchange chromatography, HiTrap SP HP, at pH 8 for AvBD1. The insect cells were infected with AvBD1 recombinant virus for 3 days and centrifuged 20 min at 5,000 x g to obtain the supernatant and the pellet. Both samples were then mixed with the start buffer (50 mM Bicine, pH8) as described in 2.19.1 Cationic exchange chromatography section. The elution was a continuous ionic gradient of 0 to 0.5 M NaCl that started from the fraction 1 and finished at the fraction 28. The flow rate was 5 ml/min with a gradient volume of 10 x column volumes and the detection was UV traces at 280 nm. The eluate was collected in 2 ml fractions. The percentage represents the antimicrobial activity of the peak's samples. The negative control allowing the calculation of the killing activity for each peak contained buffer B only (0.5 M NaCl-50 mM Bicine, pH 8).

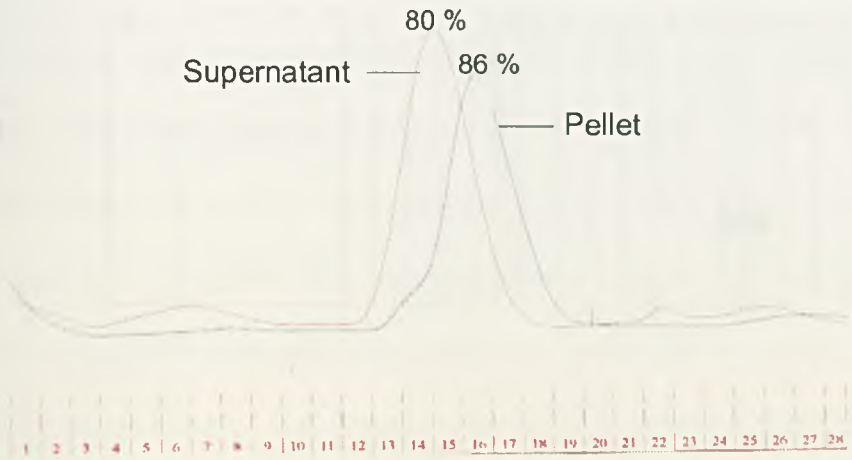
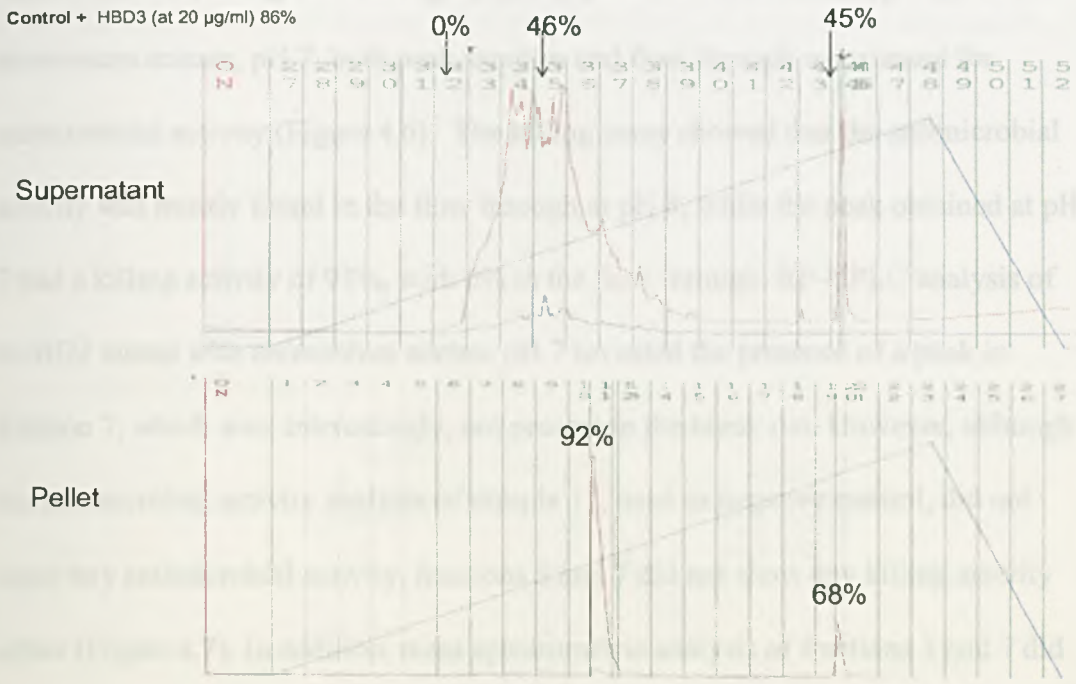


Figure 4.5: Reverse-phase HPLC fractionation of cationic peptides. The pool of fractions containing proteins after the cationic exchange chromatography were further analysed by RP-HPLC as described in 2.19.2 Reverse Phase-HPLC section. The percentage represents the antimicrobial activity of the peak's samples. The negative control allowing the calculation of the killing activity of each peak contained 0.01% acetic acid only.



the molecular weight expected for AvBD1. Two peptides of 7.9 kDa and 8.1 kDa respectively were obtained. Based on previous publications (Bals *et al.*, 1998; Valore *et al.*, 1998), the equilibration and elution buffers were changed to ammonium acetate, pH 7, to purify AvBD2 from the supernatant and the subsequent result compared to AvBD2 and 3 supernatants purified with bicine.

After cationic exchange chromatography was performed with bicine, pH 8, and ammonium acetate, pH 7, both peak samples and flow through were tested for antimicrobial activity (Figure 4.6). The killing assay showed that the antimicrobial activity was mostly found in the flow through at pH 8, while the peak obtained at pH 7 had a killing activity of 93%, with 0% in the flow through. RP-HPLC analysis of AvBD2 eluted with ammonium acetate pH 7 revealed the presence of a peak in fraction 7, which was, interestingly, not present in the blank run. However, although the antimicrobial activity analysis of sample 11, used as negative control, did not show any antimicrobial activity, fractions 5 and 7 did not show any killing activity either (Figure 4.7). In addition, mass spectrometric analysis of fractions 5 and 7 did not reveal any peptides corresponding to the molecular weight of AvBD2. Therefore, correct expression of the avian β-defensin was called into question again. The recombinant virus was therefore analysed to verify the correct insertion of the AvBD cDNA into the virus DNA.

4.3.3 Recombinant virus checking

BAC10:KO_{1629} bacmid contains a restriction enzyme cleavage site, *Bsu*36I, in ORF1629 that encodes an essential gene involved either in nucleocapsid packaging or modification of the virion RNA polymerase (Zhao *et al.*, 2003). Therefore,

Figure 4.6: Cationic exchange chromatography, HiTrap SP HP, at pH 8 for AvBDs 2 (red trace) and 3 (grey trace) and at pH 7 for AvBD2 (blue trace). The insect cells were infected with AvBD2 recombinant virus for 3 days and centrifuged 20 min at 5,000 x g to obtain the supernatant. The sample was then mixed with the start buffer (50 mM Bicine, pH8 or 32 mM ammonium acetate, pH 7) as described in 2.19.1 Cationic exchange chromatography section. The elution was a continuous ionic gradient of 0 to 0.5 M NaCl that started from the fraction 1 and finished at the fraction 28. The flow rate was 5 ml/min with a gradient volume of 10 x column volumes and the detection was UV traces at 280 nm. The eluate was collected in 2 ml fractions. The percentage represents the antimicrobial activity of the sample peaks. The negative control allowing the calculation of the killing activity of each peak contained buffer B only (0.5 M NaCl-50 mM Bicine, pH 8 or 0.5 M NaCl-32 mM ammonium acetate, pH 7).

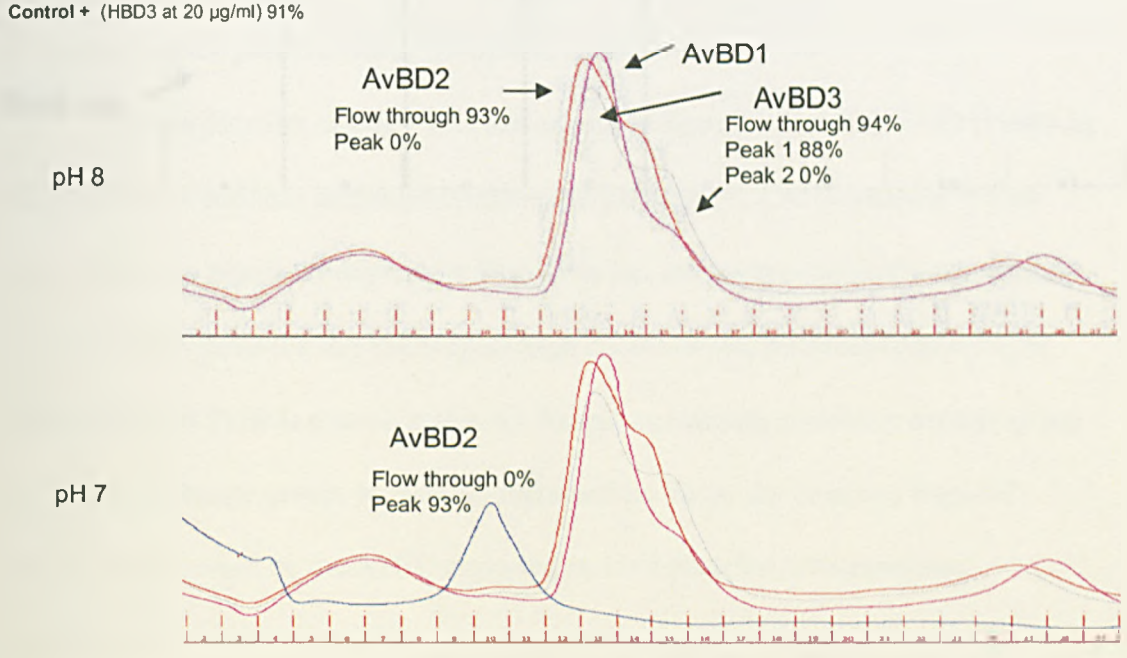
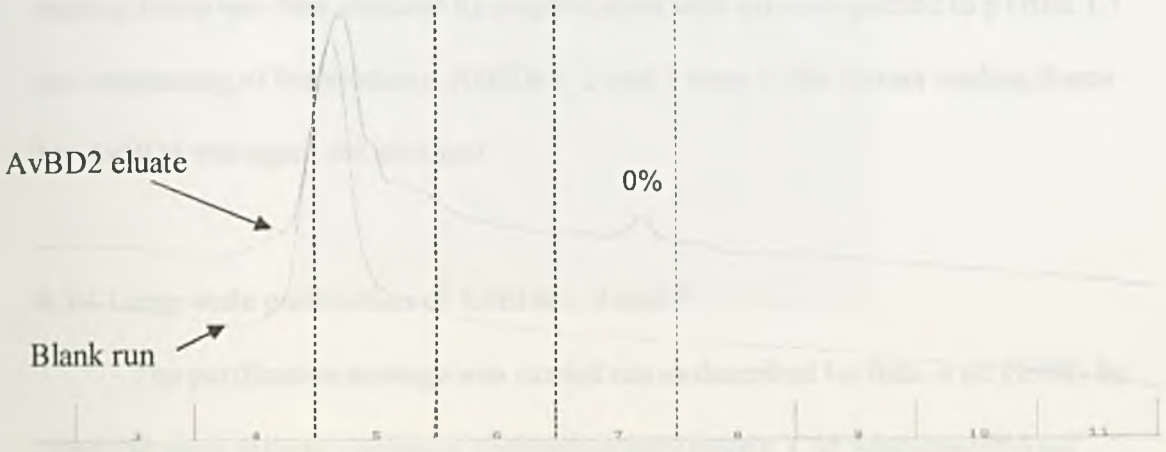


Figure 4.7: RP-HPLC of AvBD2 eluate. The pool of fractions containing proteins after the cationic exchange chromatography were further analysed by RP-HPLC as described in 2.19.2 Reverse Phase-HPLC section. The percentage represents the antimicrobial activity of the AvBD2 eluate peak's samples. The negative control allowing the calculation of the killing activity of each peak was fraction 11. Between dotted lines are the fractions used for antimicrobial assay.



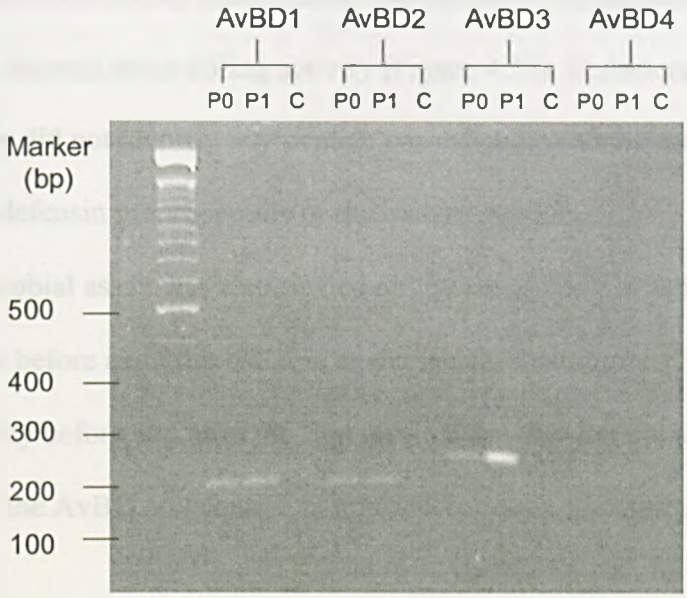
interruption of ORF1629 makes the virus non-viable *in vivo* and a viable genome can only be obtained by recombination with a suitable transfer vector, meaning that the BAC10:KO_{1629} bacmid is able to produce 100% recombinant virus following transfection (Zhao *et al.*, 2003). Despite this, the virus DNA was extracted and amplified with AvBD-specific primers to check the correct insertion of the gallinacin cDNAs into the viral genome. Bands corresponding to AvBD 1, 2 and 3 cDNAs were obtained, but AvBD4 did not yield a band (Figure 4.8). The correct reading frame was then checked by amplification with primers specific to pTriEx 1.1 and sequencing of the products. AvBDs 1, 2 and 3 were in the correct reading frame but AvBD4 was again not obtained.

4.3.4 Large-scale purification of AvBDs 1, 2 and 3

The purification strategy was carried out as described by Bals *et al.* (1998) by using the same cationic exchange chromatography matrix, CM Sepharose™ Fast Flow. The base matrix of Sepharose Fast Flow ion exchangers is highly crosslinked agarose which gives the ion exchangers high chemical and physical stability. CM Sepharose Fast Flow is a weak cation exchanger containing a carboxy methyl group as the ion exchange group. In order to differentiate the peaks obtained from RP-HPLC, which could be the AvBD expected or viral proteins, a recombinant baculovirus coding for chicken IL-22 was used as negative control. The IL-22 recombinant virus was constructed by Uday Pathania (from this laboratory) in Prof Ian Jones' laboratory at the University of Reading and was successful in producing bioactive chicken IL-22 (data not shown).

The samples from the cationic exchange chromatography were obtained after a one-step elution and no differences between the negative control eluate and the AvBDs

Figure 4.8: Electropheris gel of AvBD cDNAs from recombinant virus DNA. P0, low titre virus DNA; P1, high titre virus DNA; C, PCR control. AvBD1 (198 bp), AvBD2 (195 bp), AvBD3 (243 bp), AvBD4 (185 bp).



eluates were observed (Figure 4.9). Fractions 9 and 10 were used for RP-HPLC analysis and a few differences in the peaks obtained were observed between the control and AvBD samples (Figure 4.10). In addition, antimicrobial assays of the peaks present in the AvBD purifications and absent in the negative control purification showed some killing activity (Figure 4.10). Unfortunately, mass spectrometry did not identify any peptide corresponding to the molecular weight of the avian β-defensin prepropeptide or the mature peptide.

The antimicrobial assay was also carried out by using AvBDs and control supernatants before and after cationic exchange chromatography. The results showed killing activity before and after the first purification step but the antimicrobial activities of the AvBD and control samples were relatively similar (Figure 4.2B).

4.4 Discussion

Expression of recombinant AvBDs would allow the development of bioassays to assess their antimicrobial activity against different pathogens or their chemotactic activity for macrophages and lymphocytes, but also the production of antibodies to detect their expression in tissues. AvBD1, 2 and 3 recombinant baculoviruses were successfully produced. However, the absence of a AvBD4 band after amplification of the virus DNA excluded the AvBD4 recombinant baculovirus from further purification. Because the recombination process was well characterised and was able to produce 100% recombinant virus, the PCR of AvBD4 virus DNA was called into question. After investigation, the primers used were in fact not appropriate for the annealing temperature used for this experiment. The pTriEx 1.1 specific primers did not yield any AvBD4 products either and very low amounts of AvBDs 1, 2 and 3, suggesting a lack of PCR optimisation. Therefore, AvBD4 recombinant virus may

Figure 4.9: Cationic exchange chromatography, CM Sepharose™ Fast Flow, of AvBD 1, 2, 3 and control virus supernatants. The insect cells were infected with AvBD1, 2, 3 recombinant virus and control virus (IL-22 recombinant virus) for 3 days and centrifuged 20 min at 5,000 x g to obtain the supernatant. The sample was then mixed with the start buffer (32 mM ammonium acetate, pH 7) as described in 2.19.1 Cationic exchange chromatography section. The elution was a one step elution with 32 mM ammonium acetate/0.8 M NaCl, pH 6 buffer. The flow rate was 10 ml/min and the detection was UV traces at 280 nm. The eluate was collected in 5 ml fractions.

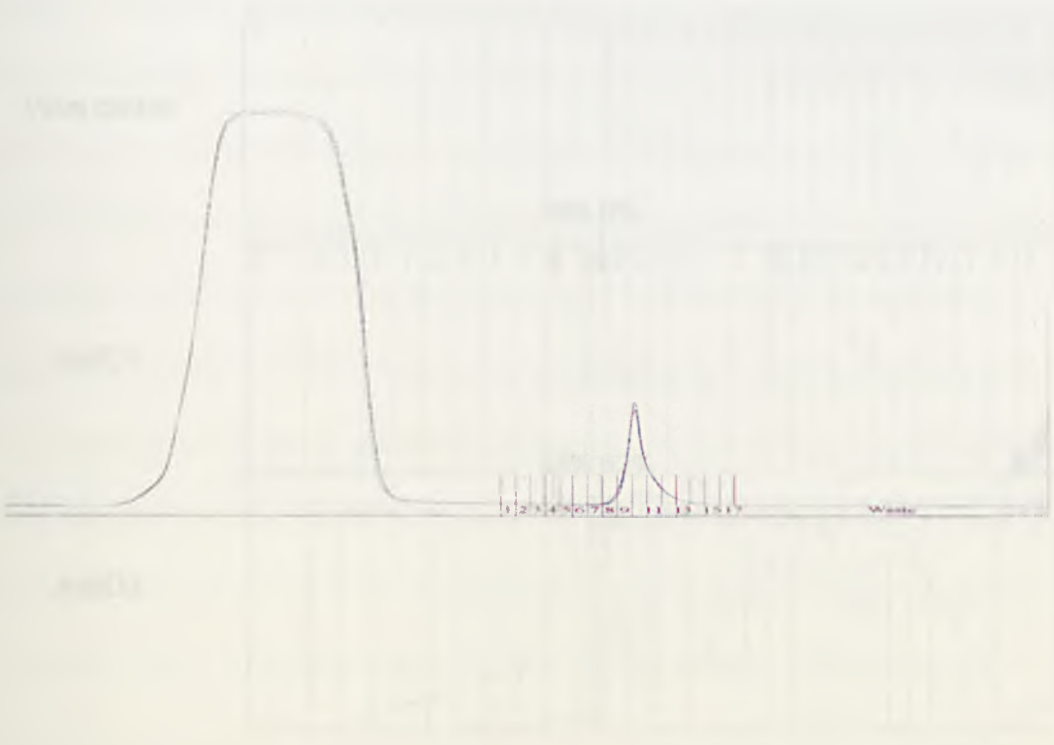
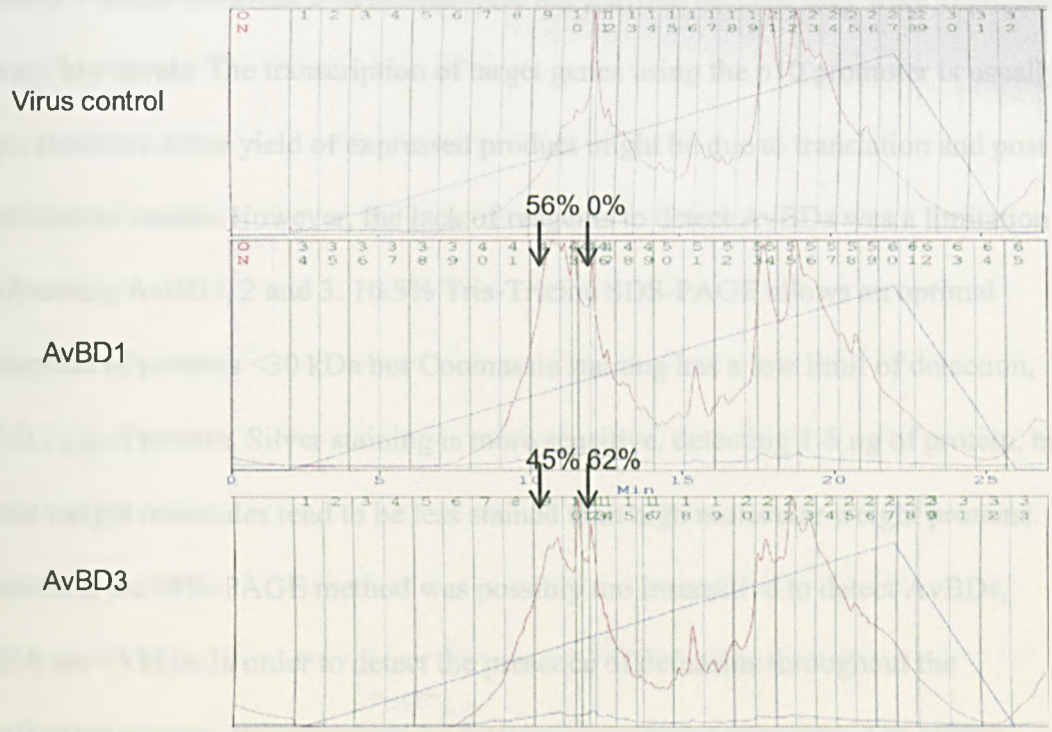


Figure 4.10: Reverse Phase-HPLC analysis of AvBD1, 3 and virus control eluates. The pool of fractions containing proteins after the cationic exchange chromatography were further analysed by RP-HPLC as described in 2.19.2 Reverse Phase-HPLC section. The virus control is the IL-22 recombinant virus. The arrows represent the sample peaks absent in the virus control analysis. The percentage represents the antimicrobial activity of the sample peaks. The negative control allowing the calculation of the killing activity of each peak contained 0.01% acetic acid only.



have been obtained successfully, as with like AvBD1, 2 and 3 recombinant viruses and an optimisation of the PCR from virus DNA would be necessary.

Although AvBD1, 2 and 3 recombinant viruses were obtained, the respective AvBD peptides were not successfully purified. The expression system used to produce AvBDs has been used previously in our lab to successfully produce chicken IL-22, with the product detected by western blot and bioactivity also demonstrated (data not shown). Perhaps the avian β-defensins were not purified because they were expressed at very low levels. The transcription of target genes using the p10 promoter is usually high, therefore a low yield of expressed product might be due to translation and post-translational causes. However, the lack of reagents to detect AvBDs was a limitation in obtaining AvBD1, 2 and 3. 16.5% Tris-Tricine SDS-PAGE allows an optimal separation of proteins <30 kDa but Coomassie staining has a low limit of detection, 0.1-0.5 μg of protein. Silver staining is more sensitive, detecting 1-5 ng of protein, but lower weight molecules tend to be less stained than high molecular weight proteins. Therefore, the SDS-PAGE method was possibly too insensitive to detect AvBDs, which are <5 kDa. In order to detect the presence of defensins throughout the purification process, the antimicrobial activity of defensins was used. The killing activity of β-defensins is non-specific via the interaction of the peptide, which is positively charged, with the bacterial membrane, which is negatively charged. Using the IL-22 recombinant virus as a control, the virus control supernatant before and after the cationic exchange chromatography showed similar antimicrobial activity to the supernatant obtained after infection with the AvBD1, 2 and 3 recombinant viruses (Figure 4.2B). Because IL-22 never demonstrated any killing activity, the antimicrobial assay indicates the inhibition of *E.coli* growth, which may be caused by any virus or insect cell protein and give false positives. For this purpose, mass

spectrometry was used to detect AvBD peptides in samples presenting antimicrobial activity. In addition, the molecular weight could indicate whether the avian β-defensin peptide was secreted in the supernatant as prepropeptide or mature peptide. Unfortunately, no AvBD forms were detected in samples with positive antimicrobial activity. Alternatives to detect expressed peptide at low yields could be considered in future, such as the addition of tags at the 3′ end of the cDNA, which can be removed after purification so as to not affect the folding and therefore the activity of the recombinant protein (Satchell *et al.*, 2003).

The characteristics of defensins limit the choice of expression and purification strategies. Their antimicrobial activity limits the choice of heterologous systems, whilst their relatively small size and the importance of the correct folding limit the purification and detection strategies. The most efficient and successful method, which tends to be more and more used, is to chemically synthetise defensins (Boniotto *et al.*, 2006; Sayama *et al.*, 2005; van Dijk *et al.*, 2007). However, lower cost methods have been recently improved, such as an *E. coli* system (Peng *et al.*, 2004). This system synthetises a new coding sequence of the target defensin using favoured codons for *E. coli*, appropriate restriction sites to clone the gene in the expression vector, a cleavage site between the propiece and the mature sequence to obtain the mature peptide and another final cleavage site at the end of the sequence to separate the peptide from the fusion protein (Peng *et al.*, 2004). Despite good levels of expression, 1.3 g/l, which were then improved to 2 g/l by using a cell-free system, the expression system remains complex and labour intensive. Interestingly, AvBD9 mature peptide was produced with success in HEK293-EBNA cells as a fusion protein with human growth hormone that facilitates the purification by using affinity chromatography and was then removed from the recombinant AvBD9 by cleavage. Unfortunately, the

antimicrobial activity of the recombinant AvBD9 was lower than that of synthetic AvBD9 (van Dijk *et al.*, 2007).

In conclusion, the production of avian β-defensin peptides remains labour intensive and/or costly, and although killing activity was demonstrated, purification of the actual defensin peptides was unsuccessful. The role of defensins in the innate immune response of the chicken was therefore examined by measuring their mRNA expression levels in a variety of *in vitro* and *in vivo* systems.

Chapter 5 – Avian β-defensin mRNA expression in chickens infected with *Salmonella*

5.1 Introduction

Salmonella enterica remains one of the most important agents of food-borne disease in man, with around 30,000 cases of salmonellosis notified per year in the UK, arising mainly from the consumption of infected poultry meat and eggs (reviewed by Barrow, 2000). The serotypes of this facultative intracellular pathogen can be divided into two groups according to the nature of the disease caused and the host specificity. The range of diseases caused depends on the host species infected and also on the expression of a variety of virulence determinants, some of which are encoded by Salmonella Pathogenicity Islands (McClelland *et al.*, 2001; Parkhill *et al.*, 2001). Despite the absence of clinical signs in adult chickens, the broad host range *S. enterica* serovars Enteritidis and Typhimurium colonise the alimentary and reproductive tracts and contaminate poultry carcasses and eggs, which enter the human food chain. The restricted host range serotypes, *S.* Pullorum and *S.* Gallinarum in the chicken, are no less invasive but do not induce a rapid inflammatory response (Henderson *et al.*, 1999; Kaiser *et al.*, 2000) and do not colonise the intestine. These bacteria can cause mortality rates of more than 50% in domestic poultry (Jones *et al.*, 2001; Shivaprasad, 2000). This ability to cause severe systemic disease seems to require interaction with the intestinal epithelia (Barrow *et al.*, 1994; Pascopella *et al.*, 1995) and may be at least in part due to the lack of a IL-1β- and IL-6-induced innate inflammatory response in the early stages of infection (Kaiser *et al.*, 2000). Therefore, host defense mechanisms play a central role in differential responses to *Salmonella* infections exhibited by different lines of chickens.

The host genetic background plays an important role in the outcome of infection. Resistance to systemic salmonellosis (Bumstead & Barrow, 1988) differs between inbred lines of chickens and at least is largely controlled by the *SAL1* locus. Lines C,

7_2 and 15I are susceptible to *S.* Enteritidis, *S.* Typhymurium, *S.* Gallinarum and *S.* Pullorum infection, while lines Wl, 6_1 and N are resistant to systemic salmonellosis (Wigley *et al.*, 2002). Inbred lines also differ in their susceptibility to intestinal colonisation with *S.* Typhimurium, although this has no relationship to the *SAL1*-mediated resistance to systemic disease, nor to the MHC (Barrow *et al.*, 2004). The susceptibility/resistance phenotype, measured as variations in the duration and amount of bacterial excretion, is expressed within 24 hours of experimental infection of six-week-old birds. The resistance is autosomal and dominant, but the responsible genes have yet to be characterised. An increase in numbers of circulating heterophils, the avian equivalent of the mammalian neutrophil, was observed in a resistant chicken line, line 6_1, following infection, suggesting the involvement of the innate immune response (Barrow *et al.*, 2004).

In the present study, inbred lines 6_1 and N, previously characterized for their resistance to systemic disease and their differences in levels of *Salmonella* colonization, were selected to analyze avian β-defensin expression. Line 6_1 and line N chickens are resistant and susceptible to *Salmonella* serovar Typhimurium colonisation respectively (Barrow *et al.*, 2004; Beal *et al.*, 2005). An important difference in response between the lines was the number and activity of circulating heterophils, suggesting the involvement of these cells and their secreted components, such as AvBDs, in resistance to gut colonization. Therefore, AvBDs 1/1α and 2, originally isolated from heterophils, were chosen to study their mRNA expression in resistant and susceptible chickens following *Salmonella* colonization. However, a role for β-defensins expressed by epithelial tissues cannot be ruled out and AvBD3 and 5 were therefore also studied.

The differential response of inbred lines to *Salmonella* serovars suggests the involvement of a common mechanism of resistance. As mentioned earlier, line 7_2 and line 6_1 are susceptible and resistant to salmonellosis. Therefore, avian β-defensin expression was also analysed in these two other lines, line 6_1 and line 7_2, following infection with different *Salmonella* serovars.

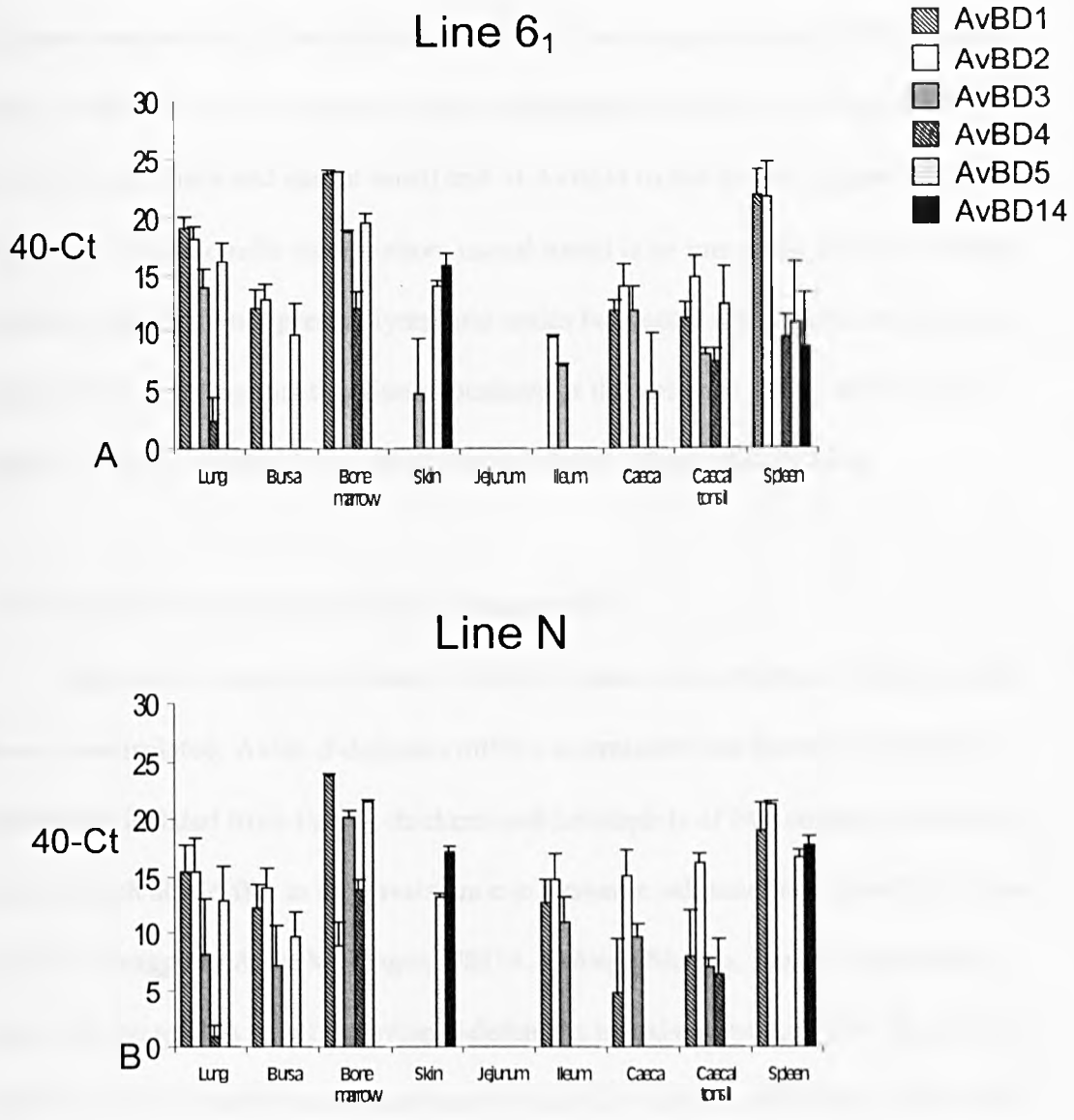
5.2 Results

5.2.1 Tissue expression profiles of AvBDs in uninfected chickens

Avian β-defensins are difficult to purify from tissues or leukocytes. They are also difficult to express in heterologous expression systems. As a consequence, bioassays to assess their function are not readily available, and there are no anti-AvBD monoclonal antibodies. However, we can analyse their expression at the mRNA level, an approach which has already been used for human and mice defensins and shown to be an appropriate reporter to analyze their differential expression in epithelial tissues (Jang *et al.*, 2004; O'Neil *et al.*, 1999; Salzman *et al.*, 2003a; Uehara *et al.*, 2003; Zaalouk *et al.*, 2004).

The avian β-defensins measured were differentially expressed in the tissues tested (Figure 5.1). Some avian β-defensins, such as AvBDs 1, 2 and 3, were widely expressed and AvBDs 1 and 2 tend to be more highly expressed than AvBD3. By contrast, AvBDs 4, 5 and 14 were expressed only in certain tissues. In line N chickens AvBD5 was not expressed in the gut but it was expressed in the caeca and caecal tonsils of line 6_1 chickens. AvBD4 was only expressed in the caecal tonsil, bone marrow and lung of both lines, and the spleen of line 6_1 chickens. AvBD14 was only expressed in the skin and spleen of both lines (Figure 5.1). In addition, some tissues,

Figure 5.1: Quantification of AvBD mRNA levels in different tissues of 6-week-old line 6_1 and line N chickens. Samples were collected as described in the section 2.12 Experimental plan. Results are expressed as corrected 40-Ct ± S.E.M. (n=3).



such as jejunum, did not express any of the avian β-defensins tested, while the bone marrow and the lung expressed all bar AvBD14. Interestingly, the skin seems to express only AvBDs 5 and 14 in both lines and a low level of AvBD3 in line 6_1 only. Finally, differences in avian β-defensin mRNA expression levels were also observed between lines. Line 6_1 chickens showed no expression of AvBDs 1 and 3 in the ileum and bursa respectively. Line N chickens showed lower expression of AvBD1 in the caeca, AvBD2 in the bone marrow and no expression of AvBD3 in the skin, of AvBD5 in the caeca and caecal tonsil and of AvBD4 in the spleen (Figure 5.1). Regarding to *Salmonella* colonization, caecal tonsil is an important tissue to analyse. Indeed, chickens do not present lymphoid nodes but caecal tonsil contains lymphoid aggregates suggesting that this tissue localized at the entrance of caecal, which is highly colonized, might play a role in the regulation of commensal flora.

5.2.2 Expression of avian β-defensins in heterophils

Heterophils were not isolated from the tissues from which the RNA isolated above, was isolated. Avian β-defensin mRNA expression was therefore assessed in heterophils isolated from line 7_2 chickens and heterophils of two commercial lines, A and B, which also differ in their resistance to systemic salmonellosis (kindly provided by Dr C. Swaggerty & Dr M. Kogut, USDA, College Station, Texas). Heterophils expressed the mRNA of all the avian β-defensins tested except AvBD14. In addition, AvBD1 was more highly expressed than AvBD3 ($P< 0.05$), AvBD4 and AvBD5 ($P< 0.005$) in line B chickens and AvBDs 4 and 5 ($P< 0.05$) in line A chickens (Figure 5.2A). However, expression of all five AvBDs was not altered in heterophils stimulated with *S.* Enteritidis compared to unstimulated heterophils (Figure 5.2B).

Figure 5.2: Quantification of avian β-defensin mRNA levels in heterophils of commercial lines, A and B, and inbred line 7_2 chickens (A) and stimulated with *S.* Enteritidis (SE) (B). The RNA samples were obtained as described by Swaggerty *et al.*, 2004. Results are expressed as corrected 40-Ct ± S.E.M. (A, A SE, B and B SE, n=3; 7_2, n=1). Asterisks represent a statistically significant difference in avian β-defensin expression between AvBD1 and AvBDs 3, 4,and 5 (* for $P<0.05$ and ** for $P<0.005$), according to the Analysis of Variance (ANOVA) general linear model test with Minitab software.

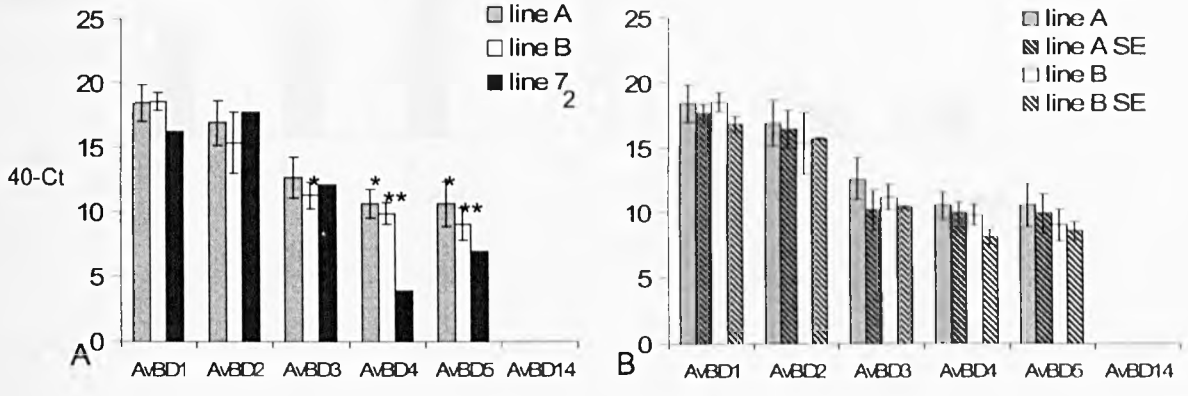
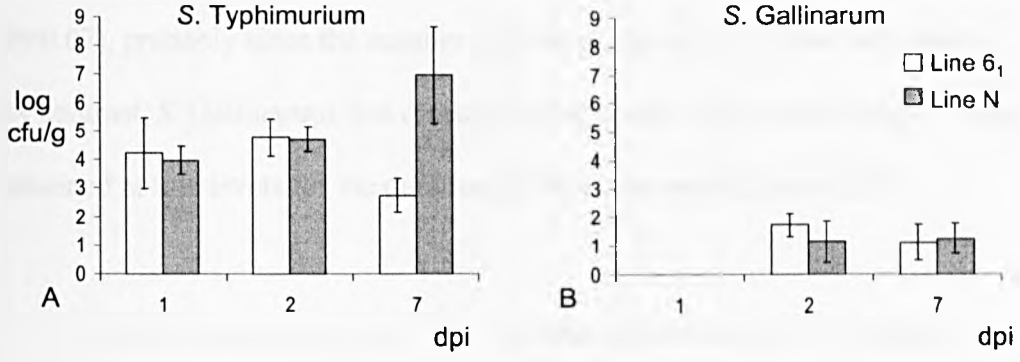


Figure 5.3: ***Salmonella*** **levels in the caeca of line 6_1 and line N chickens following oral infection with 10^8 CFU of *S.* Typhimurium (A) or *S.* Gallinarum (B).** The bacterial count is described in 2.13 Bacterial enumeration section. Results are expressed as the mean of the log CFU per g of caecal content ± S.E.M. (A, n=3; B, n=5).



5.2.3 Avian β-defensin expression in the caecal tonsils of line 6_1 and N chickens infected with *S.* Typhimurium and *S.* Gallinarum

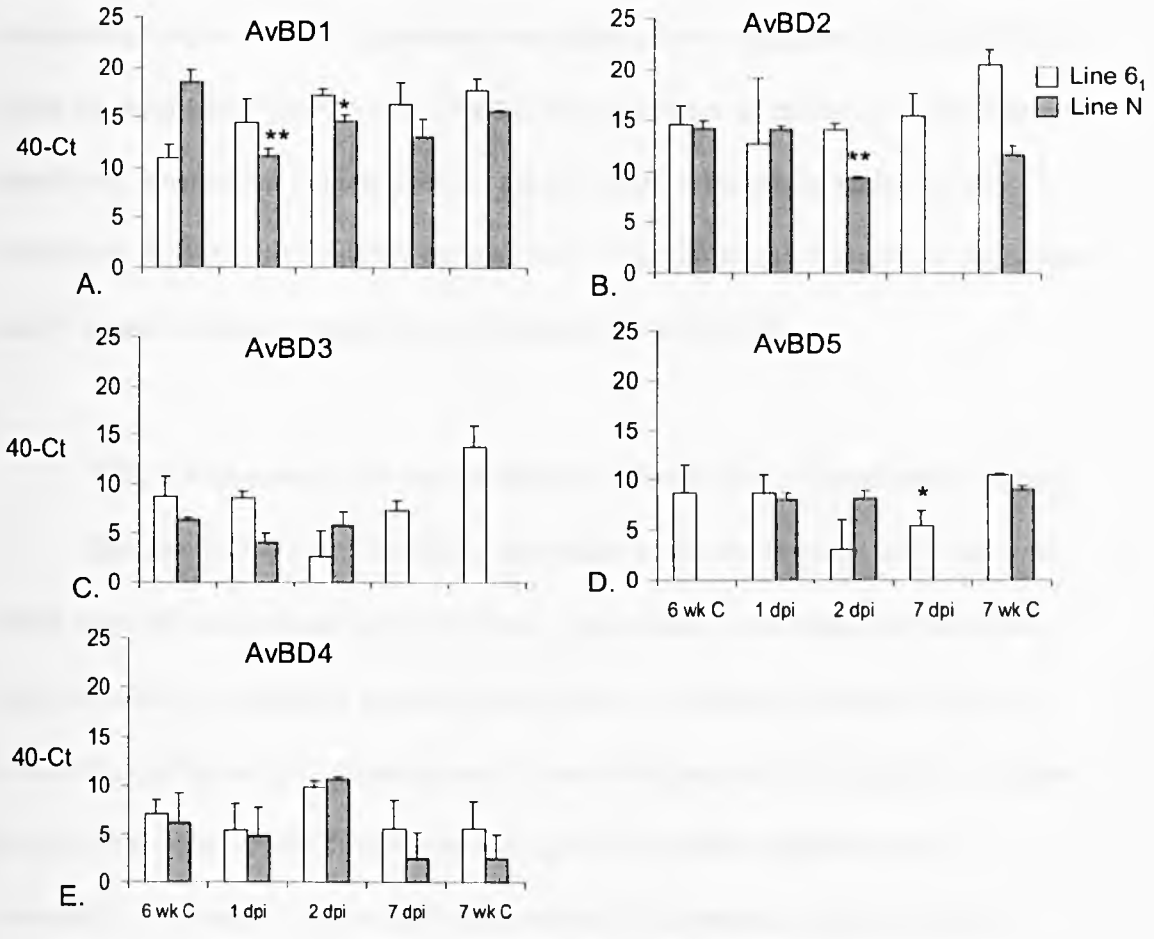
5.2.3.1 Bacterial enumeration

S. Typhimurium was detected in the caeca of line 6_1 and N chickens 1 day post-infection (dpi) (Figure 5.3A). At 7 dpi, line 6_1 chickens were clearing *S.* Typhimurium from the caeca, but the bacteria persisted in the gut of line N chickens. The differences observed between both lines were not confirmed by a statistical t-test ($P=0.07$), probably since the number of birds per group ($n=3$) was very small.
By contrast, *S.* Gallinarum was detected in the caeca of both lines from 2 dpi and remained at low levels for the duration of the experiment (Figure 5.3B).

5.2.3.2 Expression of AvBDs in chickens infected with *S.* Typhimurium

In order to determine if there is a role for avian β-defensins in the mechanisms responsible for the difference in *S.* Typhimurium colonisation observed between resistant and susceptible lines of chickens, RNA was extracted from caecal tonsils 1, 2 and 7 dpi and analysed by real-time quantitative RT-PCR. The ileum and caeca were also analysed but the low level and reproducibility of mRNA expression of some AvBDs rendered the interpretation of results difficult in both lines. The caecal tonsil gave reproducible results, as AvBDs were expressed at high levels throughout the infection.
AvBD1 mRNA was expressed throughout the infection in both lines, but it was down-regulated following infection in line N chickens, compared to levels in control birds, at 1 dpi ($P \leq 0.005$), then slightly down-regulated at 2 dpi ($P<0.05$) and finally normally expressed at 7 dpi (Figure 5.4A). AvBD2 was expressed at 1, 2 and 7 dpi in line 6_1 chickens, but only at levels seen in control birds, while in line N chickens

Figure 5.4: Quantification of avian β-defensin mRNA levels in caecal tonsils of line 6_1 and line N chickens infected with *Salmonella* Typhimurium F98. The tissue samples were collected as described in the section 2.12 Experimental plan. Results are expressed as corrected 40-Ct ± S.E.M. (n=3). Asterisks represent a statistically significant difference in avian β-defensin expression between uninfected (control) and infected chickens (* for $P<0.05$ and ** for $P<0.005$), according to Student's t-test. 6 wk C, 6 weeks old chicken uninfected; 7 wk C, 7 weeks old chicken uninfected; dpi, days post-infection.

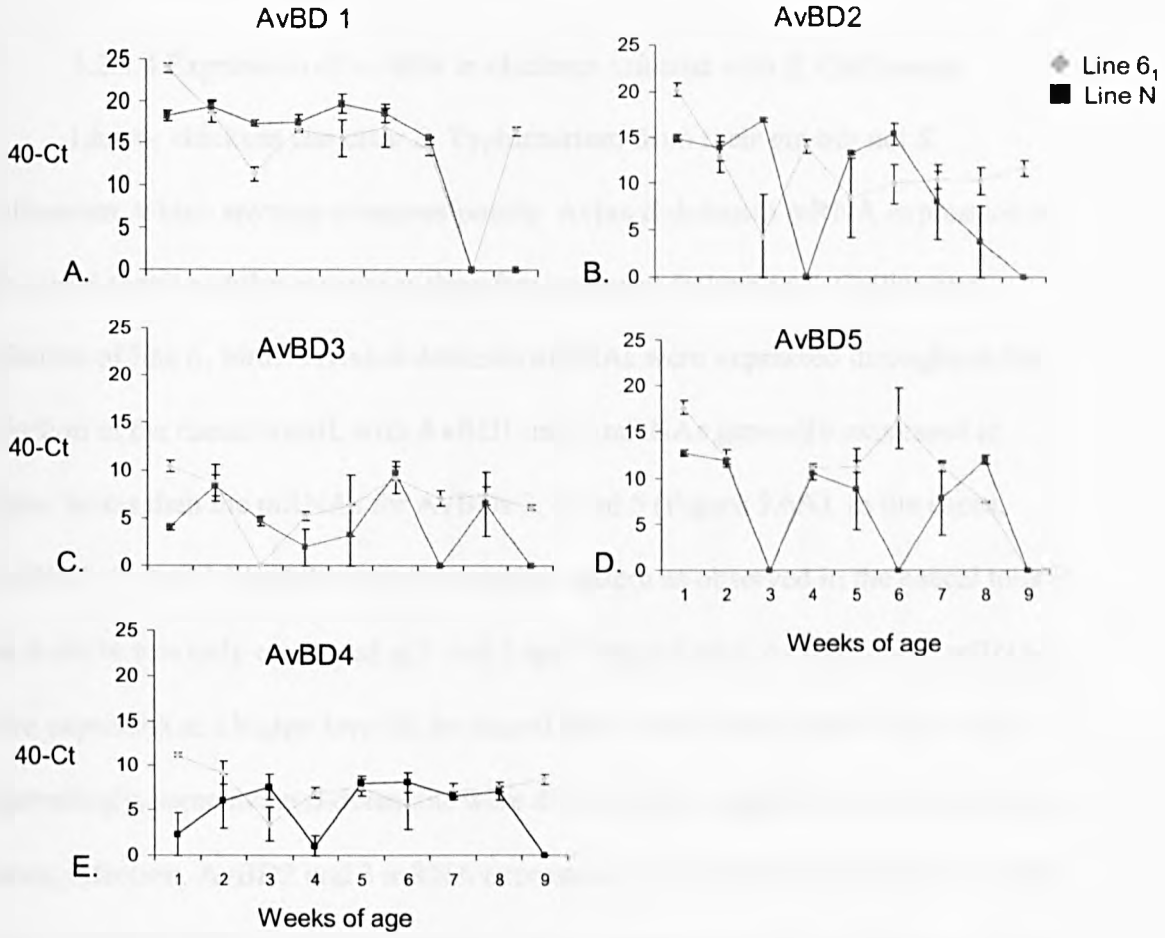


avian β-defensin2 was first normally expressed at 1 dpi, then down-regulated at 2 dpi ($P<0.005$) and finally undetectable at 7 dpi (Figure 5.4B). AvBDs 3 and 5 were normally expressed at 1 and 2 dpi in both lines but were undetectable in line N chickens at 7 dpi (Figures 5.4C and 5.4D). AvBD4 was the only avian β-defensin tested to be constitutively expressed throughout the infection (Figure 5.4E). However, down-regulation of avian β-defensin mRNA expression was not only observed in the susceptible line, as AvBD5 expression was slightly down-regulated at 7 dpi ($P<0.05$) in the resistant line (Figure 5.4D). Overall, the expression of all the avian β-defensins tested was constitutive in both lines for control birds, with the exception of the absence of AvBD3 and 5 mRNA expression in 7-week-old and 6-week-old uninfected line N control chickens, respectively (Figures 5.4C and 5.4D).

5.2.3.3 Expression of avian β-defensins in line 6_1 and N uninfected chickens

Because AvBD3 and 5 mRNA expression were not observed in 7-week-old and 6-week-old uninfected line N chickens, respectively, in the initial experiment, avian β-defensin expression was analysed weekly in uninfected chickens from 1-9 weeks of age (Figure 5.5). Expression of avian β-defensin mRNA tended to decrease in line 6_1 chickens for the first 3 weeks of age but was then expressed fairly constantly. By contrast, the avian β-defensin mRNA expression pattern in line N chickens was different for each avian β-defensin tested. AvBD1 mRNA expression in line N closely matched the expression observed in line 6_1 chickens (Figure 5.5A). AvBD2 mRNA was constantly expressed for the first 3 weeks, undetectable at 4 weeks and expression finally decreased slowly from 6 weeks of age (Figure 5.5B). AvBD3 mRNA expression tended to increase for the first 2 weeks, and then decreased slowly until 5 weeks of age but was undetectable at 7 and 9 weeks of age

Figure 5.5: Quantification of avian β-defensin mRNA levels in caecal tonsils of line 6_1 and line N chickens from 1-9 weeks of age. Samples were collected as described in the section 2.12 Experimental plan. Results are expressed as corrected 40-Ct ± S.E.M. (n=3).



(Figure 5.5C). AvBD5 mRNA expression tended to be constant but was undetectable at 3, 6 and 9 weeks of age (Figure 5.5D). Finally, AvBD4 mRNA expression increased for the first 3 weeks of age, was undetectable at 4 and 9 weeks of age and tended to be constantly expressed from 5 to 8 weeks of age (Figure 5.5E).

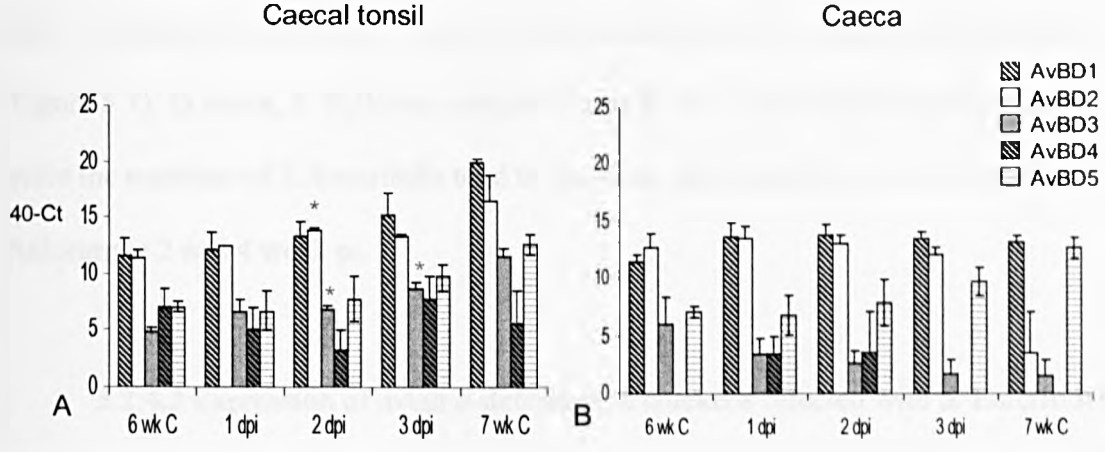
5.2.3.4 Expression of AvBDs in chickens infected with *S.* Gallinarum

Line 6_1 chickens can clear *S.* Typhimurium from their gut but not *S.* Gallinarum, which anyway colonises poorly. Avian β-defensin mRNA expression in the caecal tonsil and the caeca was therefore analysed following *S.* Gallinarum infection of line 6_1 birds. Avian β-defensin mRNAs were expressed throughout the infection in the caecal tonsil, with AvBD1 and 2 mRNAs generally expressed at higher levels than the mRNAs for AvBDs 3, 4 and 5 (Figure 5.6A). In the caeca, AvBDs 1, 2, 3 and 5 had the same expression pattern as observed in the caecal tonsil, but AvBD4 was only expressed at 1 and 2 dpi (Figure 5.6B). AvBD3 and 5 mRNAs were expressed at a higher level in the caecal tonsil than in the caeca (Figure 5.6). Interestingly, some avian β-defensins were differentially regulated in the caecal tonsil during infection. AvBD2 and 3 mRNA expression levels were up-regulated at 2 dpi, while AvBD3 mRNA expression levels were down-regulated at 7 dpi (Figure 5.5A), compared to levels in control caecal tonsils.

5.2.4 Expression of avian β-defensins in the caecal tonsil and spleen of line 7_2 chickens infected with *S.* Enteritidis and *S.* Pullorum

In comparison to line 6_1 and N chickens, which are resistant to salmonellosis, line 7_2 chickens are susceptible to *Salmonella* infection. Line 7_2 chickens were infected with two types of *Salmonella*, *S.* Enteritidis, a broad-host range serovar, and *S.* Pullorum, a host-specific serovar, to compare the innate immune response of the susceptible line.

Figure 5.6: Quantification of avian β-defensin mRNA levels in the caecal tonsil and caeca of line 6_1 chickens infected with *Salmonella* Gallinarum 9. Samples were collected as described in the section 2.12 Experimental plan. Results are expressed as corrected 40-Ct ± S.E.M. (n=3 for controls; n=5 for infected, except for AvBD4 where n=3). Asterisks represent a statistically significant difference in avian β-defensin expression between uninfected (control) and infected chickens (* for $P<0.05$), according to Student's t-test. 6 wk C, 6 weeks old chicken uninfected; 7 wk C, 7 weeks old chicken uninfected; dpi, days post-infection.



S. Enteritidis has the same phenotype as *S.* Typhimurium including gut colonization and induction of a strong immune response, but taxonomically is closely related to *S.* Gallinarum and *S.* Pullorum (Li *et al.* 1993). By contrast, *S.* Pullorum does not colonize the gut and is considered as less virulent, causing pullorum disease (diarrhea),than *S.* Gallinarum that causes typhoid fever in chickens.

5.2.4.1 Bacterial enumeration

S. Pullorum was detected in lower amounts than *S.* Enteritidis in the caeca of line 7_2 chickens 24 hours and 1 week post-infection (pi) and 1 week pi in the spleen (Figure 5.7). In caeca, *S.* Pullorum remains lower than *S.* Enteritidis 2 and 4 week pi, while the numbers of *S.* Enteritidis tend to decrease and reach the same level as *S.* Pullorum at 2 and 4 week pi.

5.2.4.2 Expression of avian β-defensins in chickens infected with *S.* Enteritidis and *S.* Pullorum

Caecal tonsil and spleen from chickens non-infected and infected with *S.* Enteritidis and *S.* Pullorum were provided by Dr P.Wigley and Ms L. Chappell. Avian β-defensin mRNA expression was therefore assessed in both the caecal tonsil and spleen at 24 hours, 1, 2, 3 and 4 weeks pi. In the caecal tonsil, AvBDs 1, 2, 3 and 5 were constantly expressed in control birds and birds infected with either serovar (Figure 5.8). A similar pattern was seen in the spleen (Figure 5.9), except that AvBD5 was up-regulated at 24 hours pi in line 7_2 chickens infected with *S.* Pullorum (Figure 5.9D). AvBD14, which is specifically expressed in the spleen and skin, did not show differential expression of mRNA levels in the spleen in response to *S.* Enteritidis and *S.* Pullorum infection (Figure 5.9E).

Figure 5.7: *Salmonella* levels in the spleen (A) and caeca (B) of line 7_2 chickens following oral infection with 10^8 CFU of *S.* Pullorum and *S.* Enteritidis. The bacterial count was carried out as described in the section 2.13 Bacterial enumeration. Results are expressed as the mean of the log CFU per g of spleen or caecal contents ± S.E.M. (n=5).

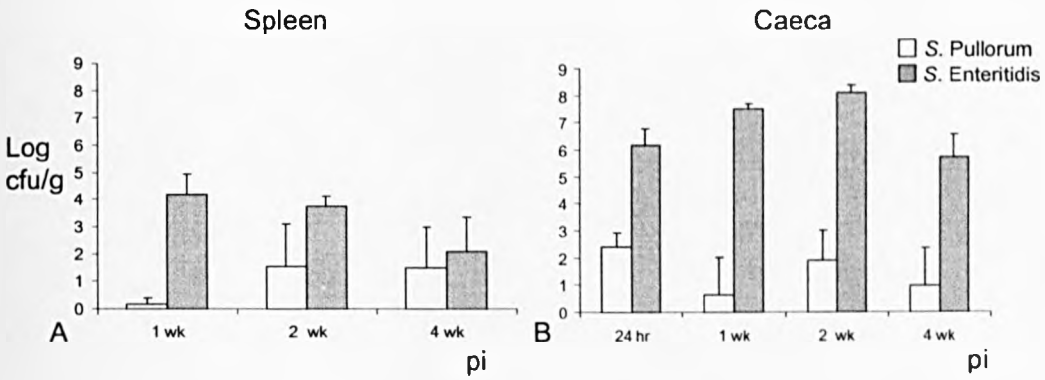


Figure 5.8: Quantification of avian β-defensin mRNA levels in the caecal tonsils of line 7_2 chickens infected with *Salmonella* Pullorum and *Salmonella* Enteritidis. Samples were collected as described in the section 2.12 Experimental plan. Results are expressed as corrected 40-Ct ± S.E.M. (n=5).

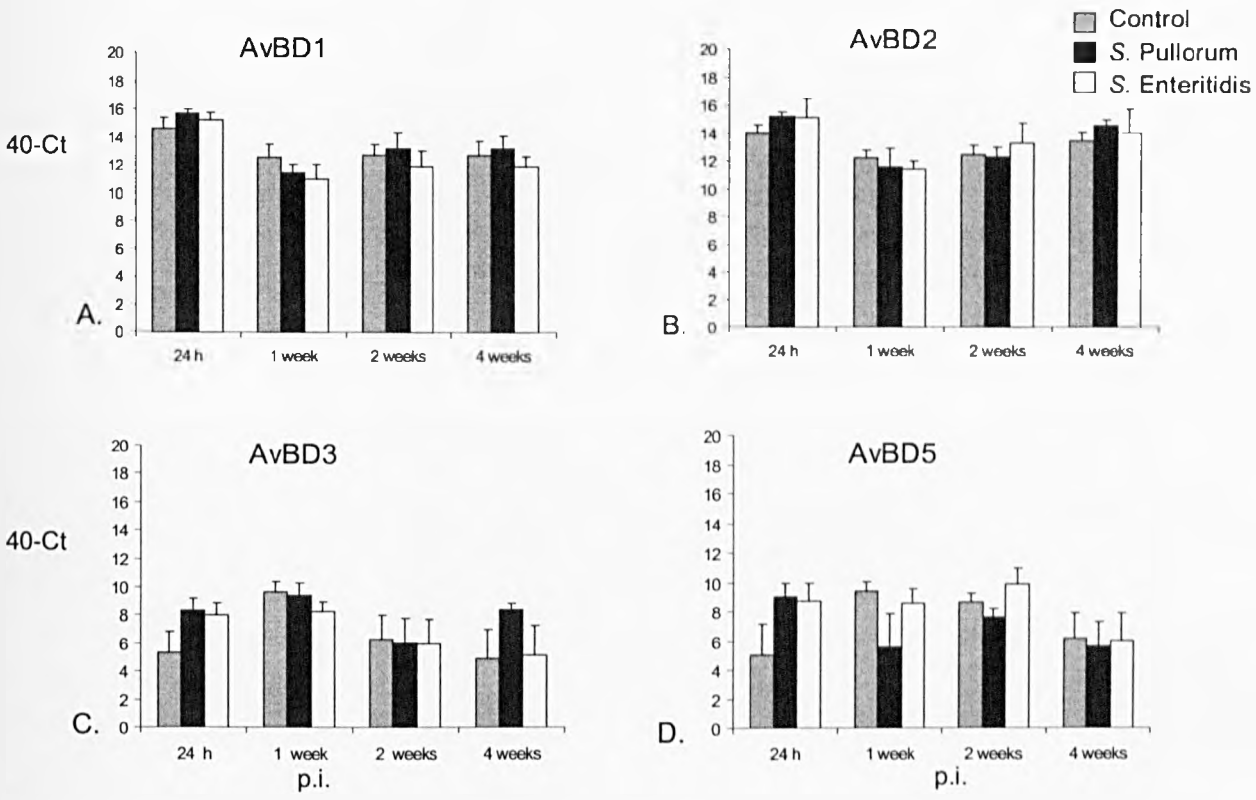
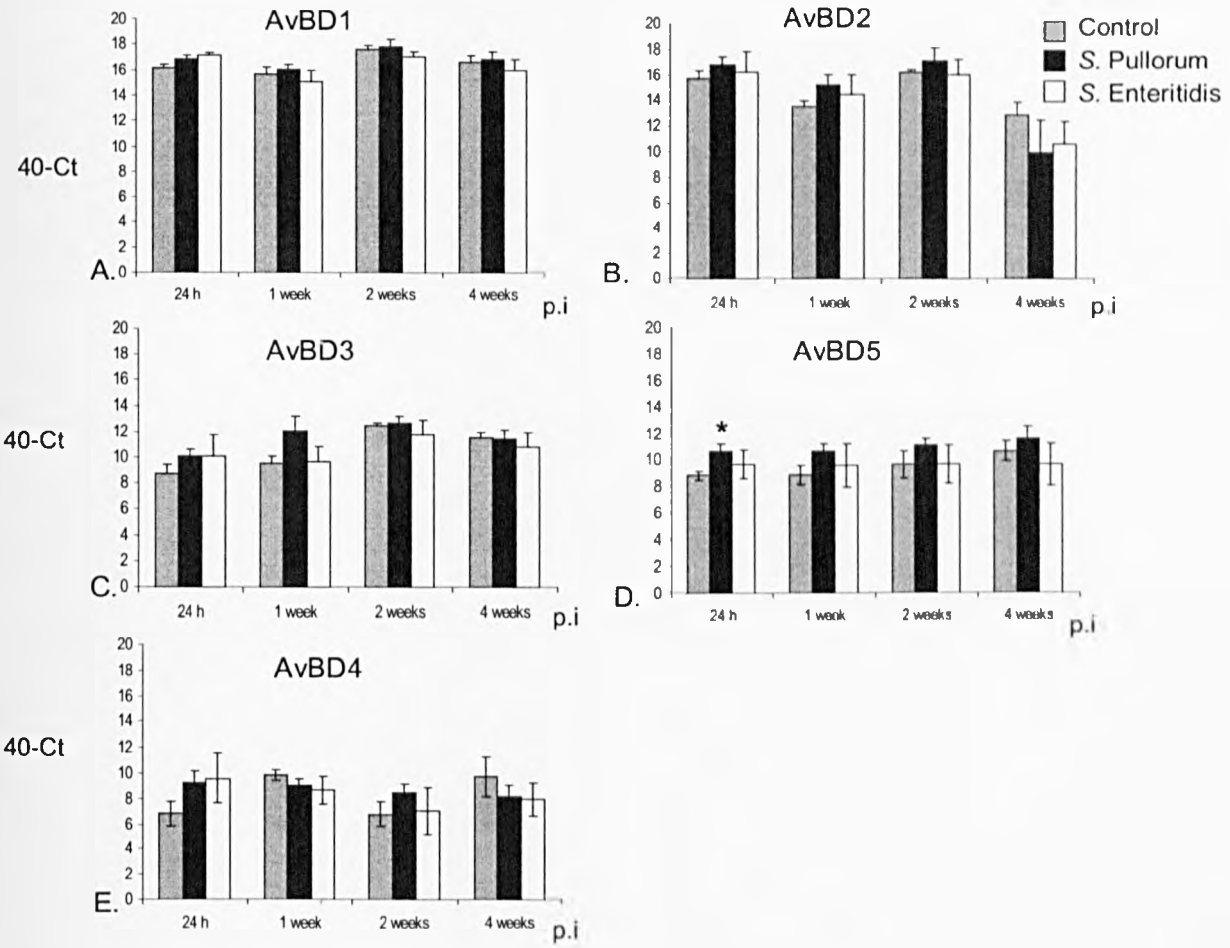


Figure 5.9: Quantification of avian β-defensin mRNA levels in the spleen of line 7_2 chickens infected with *Salmonella* Pullorum and *Salmonella* Enteritidis. Samples were collected as described in the section 2.12 Experimental plan. Results are expressed as corrected 40-Ct ± S.E.M. (n=5). Asterisks represent a statistically significant difference in avian β-defensin expression between uninfected (control) and infected chickens (* for $P<0.05$), according to Student's t-test.



5.3 Discussion

Defensins are AMPs and are an important component in mucosal host defences to prevent the invasion of enteric pathogens (Wilson *et al.*, 1999). Expression of avian β-defensin mRNA was analysed to determine their role in controlling *Salmonella* infection in the small intestine of two inbred lines of chickens showing differential susceptibility to *S.* Typhimurium colonisation, line 6_1 being resistant and line N susceptible (Barrow *et al.*, 2004). The absence of expression of AvBD2, 3 and 5 mRNAs in the susceptible line post-infection suggests that chicken defensins may play a role in the clearance of *S.* Typhimurium. AvBD5 was down-regulated in both lines. Down-regulation of defensin expression has previously been demonstrated in the mouse following infection with *S.* Typhimurium in Paneth cells (Salzman *et al.*, 2003a) and with *Cryptosporidium parvum,* a protozoan parasite, in mice intestinal epithelial cells (Zaalouk *et al.*, 2004), suggesting that suppression of epithelial defensins is a virulence strategy of small intestinal pathogens to invade the gut.

In human, most β-defensins are constitutively expressed in epithelial tissues such as the lungs, skin and intestine (Eckmann, 2005), as observed for the avian β-defensins (Lynn *et al.*, 2004; Xiao *et al.*, 2004). However, heterophils are present in the intestine of pre- and immediately post-hatch chicks, suggesting that the gut is a site for granulopoiesis (Bar-Shira & Friedman, 2006). Therefore, AvBD1 and 2, originally isolated from heterophils (Evans *et al.*, 1994), might be expressed either by gut epithelial cells and/or in heterophil granules. In addition, heterophils from different lines of chickens expressed AvBD1, 2, 3, 4 and 5 mRNAs, confirming that avian β-defensin expression seen in tissues could be from heterophils, except AvBD14. This avian β-defensin was the only defensin of those tested which was not expressed in

heterophils at the mRNA level, confirming its specificity of expression in the skin and spleen. However, mRNA expression does not reflect protein levels. Avian β-defensins can be stored in granules in heterophils and so mRNA expression of avian β-defensins does not necessarily signify their production and release in response to bacterial infection. In addition, epithelial cell expression of certain defensins in mammals alters with age (Meyerholz *et al.*, 2004; Yoshimura *et al.*, 2006). This could be a factor in the absence of AvBDs 3 and 5 expression in uninfected control birds in the first experiment at certain ages.

Avian β-defensin mRNA expression in uninfected chickens up to 9 weeks of age was different between line N and line 6_1 chickens. The regulation of defensin expression is poorly understood, even in mammals. Human β-defensin 2 (HBD-2) differs from the other human β-defensins, as it is not only constitutively expressed but also induced in response to pathogen infection or stimulation with the pro-inflammatory cytokine IL-1β (McDermott *et al.*, 2003; O'Neil *et al.*, 1999). *In vitro*, the mechanism by which IL-1β up-regulates HBD-2 involves the activation of the transcription factor NF-κB as well as activation of signaling proteins, particularly PKC, p38 MAPK, JNK and PI3K (Jang *et al.*, 2004). The ERK pathway is involved in hBD-2 and -3 expression and JNK plays a crucial role in defensin expression in response to *Helicobacter pylori* (Boughan *et al.*, 2006). In mice, the p38 MAPK pathway is also involved in the production of α-defensins (Salzman *et al.*, 2003a). The differential expression of avian β-defensins between lines and the differential expression pattern of the avian β-defensins studied in the susceptible line might in part explain the susceptibility of line N chickens to *S.* Typhimurium colonization. Indeed, AvBD1 and 2 mRNA expression increased during the first two weeks of life of unvaccinated Ross broiler chicks (Bar-Shira & Friedman, 2006), suggesting that avian β-defensin expression patterns depend

on the chicken line studied. In addition, line N chickens also showed in previous studies a decrease in number and circulating heterophils (Barrow *et al.*, 2004) and a low antigen-specific proliferation of splenocytes, suggesting the involvement of T cell responses in the clearance of *S.* Typhimurium in this resistant line (Beal *et al.*, 2005). This suggests that the genetic background controlling the innate immune response of line N chickens is presumably the main cause of their susceptibility to *S.* Typhimurium colonisation.

S. Gallinarum is a host-specific serovar causing fowl typhoid in poultry. This bacterium is less invasive than *S.* Typhimurium and does not trigger an inflammatory response (Kaiser *et al.*, 2000), thereby presumably allowing the development of systemic disease. Line 6_1 chickens are resistant to *S.* Typhimurium colonization but susceptible to *S.* Gallinarum infection. However, avian β-defensins were expressed constitutively during both infections suggesting that the killing activity of these peptides is not essential to clear the pathogen. Indeed, if the antimicrobial activity of avian β-defensins was responsible for the clearance of *S.* Typhimurium, *S.* Gallinarum could be expected to repress avian β-defensin expression to be able to cause systemic disease. In addition, the bactericidal activity of human defensins is inhibited in physiological fluids and solutions at high salt concentrations (Bals *et al.*, 1998; Goldman *et al.*, 1997), suggesting that defensins may have other biological functions. HBD-2 chemoattracts immature dendritic cells and memory T cells (Yang *et al.*, 1999) as well as inducing a cytokine response, particularly IL-6, IL-8 and IL-10 (Boniotto *et al.*, 2006), thereby linking the innate and adaptive immune responses. Because of these possible roles for defensins in the immune system, it was not surprising that mammalian defensins, such as porcine β-defensin 2, HBD2 and α-defensins, are differentially regulated in response to *Salmonella* infection *in vitro* and

in vivo (O'Neil *et al.*, 1999; Salzman *et al.*, 2003a; van Dijk *et al.*, 2006). Therefore, the slight up- and down-regulation of certain avian β-defensins observed during infection with both *Salmonella* serovars suggests that, like mammalian defensins, they may have additional roles in the innate immune response.

Avian β-defensin mRNA expression was also analysed to compare the effects of *S.* Enteritidis and *S.* Pullorum infection in line 7_2 chickens, which are susceptible to salmonellosis. *S.* Enteritidis, like *S.* Typhimurium, rarely produces disease in chickens and colonises the gastrointestinal tract, while *S.* Pullorum mainly causes clinical disease in young chickens. In addition, the colonisation pattern of host-specific *Salmonella* and broad host-range serovars are relatively similar in chicken lines resistant (line N) and susceptible (line 7_2) to salmonellosis, with higher amounts of *S.* Typhimurium and *S.* Enteritidis than *S.* Gallinarum and *S.* Pullorum in the chicken gut. The constant expression of avian β-defensin mRNA in both the caecal tonsil and spleen showed that the AMPs were not a major component in the differential infection of *Salmonella,* confirming the results with *S.* Gallinarum and *S.* Typhimurium in line 6_1 chickens, which are resistant to salmonellosis. However, the time-points selected might not be appropriate, as gallinacin 9 was up-regulated at 24 hours pi but a 48 hours time-point was missing, while the differential expression of avian β-defensins tended to be between 24 h and 7 days pi in the previous experiments. Heterophils are an important component in controlling *S.* Enteritidis infections (Kogut *et al.*, 1994) and they play a crucial role in the response to salmonellosis (Henderson *et al.*, 1999). However, the lack of differential avian β-defensin mRNA expression between resistant and susceptible lines in response to salmonellosis coincides with the lack of difference in heterophil phagocytic activity previously observed (Wigley *et al.*, 2002), suggesting a minor role for heterophils in the resistance and susceptibility of chicken

lines to salmonellosis. However, there is differential expression of cytokine and chemokines following phagocytosis of *S.* Enteritidis by heterophils (Kogut *et al.*, 2003). In addition, heterophils from commercial lines, line A and line B, respectively resistant and susceptible to *S.*enteritidis-induced mortality, showed differential expression of the pro-inflammatory cytokines IL-6 and IL-18, the pro-inflammatory chemokine CXCLi2 and the anti-inflammatory cytokine TGF-β4. Interestingly, the pro-inflammatory cytokines and chemokine were up-regulated in the resistant line, while the anti-inflammatory cytokine was down-regulated (Swaggerty *et al.*, 2006). Line A and B heterophil samples were also tested for avian β-defensin expression in this study, but no difference in avian β-defensin expression between the resistant and susceptible lines was observed, suggesting that avian β-defensins expressed by heterophils do not play a role in resistance to salmonellosis. In contrast, the up-regulation of the anti-inflammatory cytokine, TGF-β4, was also observed in the spleen of line N chickens (Beal *et al.*, 2004) suggesting that the up-regulation of TGF-β4 may increase susceptibility to *Salmonella* infection.

In summary, we observed differential expression of certain avian β-defensins in response to *Salmonella* infection. As human β-defensins are an important component of the innate immune system, it will be interesting to investigate the regulation of avian β-defensin expression and their biological functions in the innate immune response to further understand the chicken's response to *Salmonella* infection.

Chapter 6 - Discussion

6.1 Defensins are components of innate immunity in the gastrointestinal tract

Defensins are important components of innate immunity, playing multiple roles such as having antimicrobial activity against a variety of microorganisms, recruiting phagocytic cells, activating adaptive immunity by recruiting T cells and immature dendritic cells and inducing dendritic cell maturation (Yang *et al.*, 2004). Regulation of innate immunity is complex and involves cells, mediators and effector molecules that specifically recognise a pathogen and remove it. However, when the innate components are unable to stop the infection, they can also stimulate adaptive immunity to induce a strong response. The specificity of pathogen recognition is even more important in tissues colonised by commensal bacteria such as the intestinal tract. Indeed, the mammalian gastrointestinal tract contains Paneth cells that release AMPs constitutively on stimulation with bacterial components, such as LPS, lipoteichoic acid, lipid A and muramyl dipeptide (Ayabe *et al.*, 2000), to limit the invasion and adherence of pathogenic and commensal bacteria (Wehkamp *et al.*, 2007). Paneth cells localised in the base of the small intestinal crypts of Lieberkühn are granulated cells that protect stem cells, which give rise to other cell lineages including enterocytes, goblet cells and enteroendocrine cells (Bry *et al.*, 1994; Cheng & Leblond, 1974). The mechanism leading to stimulation of Paneth cells to degranulate and thereby secrete lysozyme (Erlandsen *et al.*, 1974), defensins (Cunliffe *et al.*, 2001) and secretory phospholipase A2 (Nevalainen *et al.*, 1995), by direct interaction with microorganism components or via other epithelial cells, is still unclear. However, the degranulation event is independent of TLR4 signalling components in TLR4-deficient mice (Tanabe *et al.*, 2005), while TLR9 expressed by Paneth cells is involved in degranulation when stimulated with its ligand, CpG DNA (Rumio *et al.*, 2004). In addition, a TLR-independent signalling pathway, the intracellular sensing

system of NOD proteins, was discovered to be expressed in Paneth cells, and NOD2-deficient mice showed a reduction of α-defensin expression by Paneth cells (Kobayashi *et al.*, 2005), suggesting a complex regulation between the different signalling pathways to control the degranulation of Paneth cells in response to lumenal bacterial components.

In the chicken, the presence of Paneth cells in the intestine and the caeca remains to be determined. Chicken heterophils, the functional equivalent of mammalian neutrophils, can be observed in the intestine of pre- and immediately post-hatch chicks (Bar-Shira & Friedman, 2006). The development of the gut and its colonisation is different between species that forage an adult type diet compared to others that are fed with the parents' milk (Turk, 1982). Chicken heterophils may contribute to the control of commensal flora at the early stage of the gut development particularly in the caeca, which is a major site of bacterial colonisation (Barnes, 1979), as they are able to divide outside the bone marrow and to produce mediators and effector molecules such as cytokines, chemokines, presenilin 1 and β-defensins (Bar-Shira & Friedman, 2006). In contrast, in mammals neutrophils have their origin in multi-potencial stem cells in bone marrow and migrate from the blood stream to the gut in response to infection (Hachicha *et al.*, 1998). Like Paneth cells, heterophils express a wide panel of TLR mRNAs, including TLR1/6/10, TLR2 type 1, TLR2 type 2, TLR3, TLR4, TLR5, and TLR7, which were functionally active in response to their agonists (Kogut *et al.*, 2005). Paneth cells and human neutrophils express α-defensins, while chicken heterophils express β-defensins. AvBD1 and 2 peptides were first isolated from heterophils and this study has shown that AvBD3, 4 and 5 mRNAs are also expressed by heterophils, suggesting that heterophils are an important component in chicken gastrointestinal immunity.

Epithelial cells are a natural barrier limiting the invasion of commensal and pathogen bacteria. In mammals, gastrointestinal cells express AMPs such as β-defensins, constitutively or in response to infection (Ganz, 2003b). In chickens, AvBDs are also expressed constitutively or induced by pathogens, such as AvBD4 in the small intestine, particularly the caeca and caecal tonsil, suggesting a protective role for gallinacins in the epithelial integrity of the chicken intestinal tract.

6.2 AvBD expression and genetic organisation

Heterophils express all the avian β-defensins tested, except AvBD14. AvBD14 was identified in BAC bW094K17 Contig 14 in our laboratory. This BAC also encodes AvBDs 1, 3, 4 and 5. However, AvBD14, which seems to be composed of two exons, is not fully sequenced in the chicken genome. Thirteen genes coding for AvBDs were identified on chicken chromosome 3. The genetic organisation showed similar features with the human and mice defensin loci. Indeed, the *CTSB* gene, coding for Cathepsin B, and a human EST sequence were localised on either side of the chicken defensin cluster. These conserved genes were first identified in defensin gene clusters on human chromosome 8p22 and mouse chromosome 14C3 (Xiao *et al.*, 2004). It would be interesting to sequence BAC bW094K17 fully and to align it with the avian β-defensin locus in the chicken genome to fill the gaps between contigs 17.130, 17.131 and 17.132 (Figure 3.5).

Expression of avian β-defensin mRNAs was constitutive in a variety of tissues including lung, bursa, bone marrow, spleen, skin (for AvBD5 and 14), ileum, caeca and caecal tonsil. However, the absence of monoclonal antibodies to AvBDs limits the development of bioassays to visualise the secretion of avian β-defensins by tissues, particularly in the intestinal tract. Indeed, defensins secreted by neutrophils,

heterophils and Paneth cells are stored in granules before being released, whilst the storage or direct secretion of defensins by epithelial cells remains to be determined. The mRNA level of defensins does not therefore necessarily reflect secretion of the defensin peptide into the lumen.

Because of the characteristics of defensins, the production of defensin peptides requires particular expression methods. The defensin genes encode a prepropetide, which is then cleaved to obtain a mature peptide able to attach to and form a pore in the cell membrane of the target microorganism. In addition, the correct folding of defensins depends on the formation of three disulphide bridges, which is difficult to achieve using *E. coli* expression systems. Systems that produce the mature peptide synthetically are mostly used because of their efficiency in obtaining high levels of biologically active defensins. Baculovirus expression systems have been used several times to produce human β-defensin peptides by inserting a cleavage site between the propiece and defensin mature peptides, but interestingly the baculovirus system also expressed the mature peptide directly. However, the low level of active defensin obtained limited its detection and purification was labour intensive. Recently, AvBD9 mature peptide was successfully expressed in mammalian HEK293-EBNA cells as a fusion protein with human growth hormone, which facilitated purification using affinity chromatography and was then removed from the recombinant AvBD9 by cleavage (van Dijk *et al.*, 2007). Unfortunately, the antimicrobial activity of the recombinant AvBD9 was lower than that of synthetic AvBD9. The sequence coding for the mature peptide was directly inserted in the expression vector by modifying the N-terminal of AvBD9. Despite the presence of a higher number of positive charges on the surface of the recombinant AvBD9 than the synthetic AvBD9, the modification of the recombinant AvBD9 N-terminal affected its antimicrobial activity (van Dijk *et al.*,

2007). The success of AvBD9 production, using a relatively simple expression and purification system, should allow the successful production of other avian β-defensins by cloning the avian β-defensin full cDNA sequence with a cleavage site between the propiece and the mature peptide, as previously carried out for human β-defensins produced in a baculovirus expression system (Bals *et al.*, 1998; Goldman *et al.*, 1997). This construct would produce a high level of purified biologically active avian β-defensin.

Defensins were first identified as AMPs by their microbicidal activity against a variety of microorganims such as bacteria and viruses. However, complementing roles in immune defense were observed for both α- and β-defensins. Indeed, a mouse α-defensin, cryptdin 3, secreted by Paneth cells promotes ion fluxes in epithelial cells (Merlin *et al.*, 2001) and induces expression of the chemokine IL-8 (Lin *et al.*, 2004). A human β-defensin, HBD2, was identified as being chemoattractant for dendritic cells (Yang *et al.*, 1999). In addition to their involvement in immune defence, defensins are constitutively expressed or induced in response to infection or inflammation. HBD1 is constitutively expressed in the colon, whilst HBD2 is produced in inflammatory bowel disease (O'Neil *et al.*, 1999; Wehkamp *et al.*, 2003a) or in response to bacterial infections, such as with *C. jejuni* (Zilbauer *et al.*, 2005) or *H. pylori* (Wehkamp *et al.*, 2003b). The different biological activities and expression patterns of the multiple defensins may suggest that each defensin is produced either to regulate the commensal flora by using its antimicrobial activity or to modulate immune defence in response to pathogen infection. For this purpose, the production of avian β-defensin peptides could help to define first their antimicrobial and chemotactic activity and then their specific role in immune defence in response to a variety of pathogens such as *Salmonella*.

6.3 Avian β-defensin expression in response to *Salmonella* infection

Chickens can be infected either by broad host range *Salmonella* serovars, such as *S.* Typhimurium and *S.* Enteritidis, or host-specific serovars, such as *S.* Pullorum and *S.* Gallinarum, which cause systemic salmonellosis. Previous studies of inbred White Leghorn chicken lines showed differences in resistance and susceptibility to *Salmonella* infection. Susceptible chickens, including lines 7_2, C and 15I, had a higher mortality and morbidity rate than resistant chickens, including lines Wl, N and 6_1. On post-mortem examination large necrotic lesions were observed in susceptible birds. Resistance to salmonellosis was determined to be genetically dependent and at least in part encoded by the *SAL*1 locus (Wigley *et al.*, 2002). Different lines of commercial birds, lines A and B, also showed differential resistance to *S.* Enteritidis infections, with line A more resistant to systemic *S.* Enteritidis infection than line B. Fewer line A chickens died after *S.* Enteritidis infection compared to line B and more heterophils migrated to the site of infection in the resistant line (Swaggerty *et al.*, 2005). Heterophils, as discussed above, are equivalent to mammalian neutrophils, and are an important component of innate immunity as they are able to modulate the inflammatory response through the phagocytosis of infectious agents (Desmidt *et al.*, 1996; Kogut *et al.*, 2001) and the secretion of cytokines and chemokines (Kogut *et al.*, 2003). Heterophils from line A and B chickens showed differential expression of the pro-inflammatory cytokines and chemokines IL-6, IL-18 and CXCLi2 and the anti-inflammatory cytokine TGF-β4. Interestingly, the pro-inflammatory cytokines and chemokine were up-regulated in the resistant line, while the anti-inflammatory cytokine was down-regulated (Swaggerty *et al.*, 2006). In this study, line A and B heterophil samples were also tested for avian β-defensin expression, but no difference in avian β-defensin expression between the resistant and susceptible lines was

observed, suggesting that AvBDs expressed by heterophils do not play a role in the resistance to salmonellosis.

A second component of the cellular innate immune response, macrophages, showed differential effectiveness between the resistant chicken line Wl and the susceptible line 7_2. Macrophages from the resistant line showed a stronger oxidative response to *Salmonella* compared to macrophages from the susceptible line (Wigley *et al.*, 2002). In addition, the pro-inflammatory chemokines CCLi2 and CXCLi1 were up-regulated macrophages from the resistant line challenged with *S.* Gallinarum and *S.* Typhimurium (Wigley *et al.*, 2006). Interestingly, IL-6 and IL-18 expression were also higher in macrophages from the resistant line than those from the susceptible line following challenge with both serovars (Wigley *et al.*, 2006), as observed previously in heterophils (Swaggerty *et al.*, 2006). IL-18 combined with IL-12 initiates Th1 adaptive responses (Mastroeni & Menager, 2003), which are involved in the clearance of intracellular pathogens such as *Salmonella*, *Mycobacteria* and trypanosomes (Holscher, 2004). The cellular innate components, macrophages and heterophils, from the resistant-line chickens respond therefore more effectively and rapidly in the initiation of adaptive immune responses (Wigley *et al.*, 2006), which could explain the clearance of *Salmonella* in the spleen and liver of the resistant line as previously observed (Wigley *et al.*, 2002). However, the clearance of *Salmonella* from the resistant line might be also caused by an increase of macrophage antibacterial activity through the expression of AvBDs. The expression of avian β-defensins by chicken macrophages remains to be determined but human macrophages express β-defensin 1 and 2 mRNAs (Duits *et al.*, 2002). Despite the absence of differential expression of AvBDs in heterophils of line A and B chickens, it could be interesting to analyse the

expression of AvBDs in macrophages from resistant and susceptible chicken to determine their role, if any, in chicken systemic salmonellosis.

Interestingly, chicken lines resistant to salmonellosis can also show differential susceptibility to S. Typhimurium colonisation. When chickens were infected at 6 weeks of age, line 6_1 chickens were able to clear S. Typhimurium from the intestinal tract, while the bacterium tends to persist in the gut of line N chickens. The AvBD expression analysis showed the absence of AvBD2, 3 and 5 mRNA expression in the susceptible line post-infection, suggesting that chicken defensins may play a role in the clearance of *S.* Typhimurium. Down-regulation of defensin expression has previously been demonstrated in the mouse following infection with *S.* Typhimurium in Paneth cells (Salzman *et al.*, 2003a) and with *C. parvum,* a protozoan parasite, in mice intestinal epithelial cells (Zaalouk *et al.*, 2004), suggesting that suppression of epithelial defensins is a virulence strategy of small intestinal pathogens to invade the gut. However, avian β-defensin mRNAs were also analysed in heterophils, which are an important component of the innate immune response involved in the chicken resistance to salmonellosis. In addition, heterophils were discovered in the intestine of pre- and immediately post-hatch chicks, suggesting their involvement in the control of intestinal microorganisms (Bar-Shira & Friedman, 2006). The absence of avian β-defensin expression in the line N chickens may be therefore at the level of heterophils or intestinal epithelial cells. For this purpose, heterophils from resistant and susceptible chicken lines could be isolated and challenged with *S.* Typhimurium to determine whether the down-regulation of AvBD mRNA expression observed was specifically in heterophils. Determination of how avian β-defensin expression is regulated might help understand chicken resistance to salmonellosis and to *S.* Typhimurium colonisation. Several mammalian TLRs are involved in the expression

of defensins and, interestingly, heterophils express a wide panel of TLRs. In addition, IL-18, which is important in initiating an inflammatory response, is up-regulated in heterophils and macrophages of chicken lines resistant to salmonellosis. Interestingly, human intestinal epithelial cells express IL-18 and up-regulate interleukin expression in response to *C. parvum*. The presence of the IL-18R, expressed by IECs, suggested the involvement of IL-18 epithelial host defence during infection and was confirmed *in vitro* by inducing the expression of α-defensin 2 and LL-37, but not α-defensin 3 (McDonald *et al.*, 2006). Therefore, it will be interesting to analyse the effect of IL-18 on avian β-defensin expression in chickens resistant to salmonellosis.

6.4 Future prospects for defensin research

Antimicrobial molecules are ancient small cationic molecules encoded by the host that are considered as antibiotic-like effectors of innate immunity. They are composed of inorganic disinfectants (e.g. hydrogen peroxide and nitric oxide), small AMPs (e.g. defensins and cathelidicins) and large AMPs (e.g. lysozyme and phospholipase A_2) (Yang *et al.*, 2002). Defensins display antimicrobial activity against a wide range of bacteria, fungi and viruses, and are considered as effectors of innate antimicrobial immunity. However, defensins are more than just antibacterial in immunity and are able to chemoattract a variety of inflammatory, immune and other cell types, including neutrophils, macrophages, monocytes, lymphocytes and dendritic cells *in vitro* and *in vivo*, thereby contributing as immunological adjuvants to the activation and regulation of adaptive immunity against pathogen infection (Yang *et al.*, 2002). By binding chemokine receptors such as CCR6, murine β-defensin 2 and 3, fused with nonimmunogenic tumor antigens, have yielded potent antitumor vaccines. Indeed, the induction of an adaptive response to weakly immunogenic tumor antigens

could be enhanced by fusion with β-defensins to target the delivery of such antigens to receptors of APCs, particularly immature dendritic cells, to obtain protective antitumor immunity (Biragyn *et al.*, 2001). The fusion of murine β-defensin 2 and 3 with a B cell lymphoma epitope and used as DNA vaccines in mice, generated potent humoral responses and the successful development of antitumor immunity. In addition, the involvement of receptors other than CCR6 is not excluded, as the murine β-defensin 2 and 3 fusion constructs generated different immune responses. The murine β-defensin 3 fusion construct generated higher antibody titres than the murine β-defensin 2 fusion construct, but did not induce antitumor immunity, unlike murine β-defensin 2 in one of the models tested, the A20 tumor model. However, the use of chemokine fusion constructs that selectively activate immature dendritic cells induced protection, particularly chemokines that activate CCR6 on immature dendritic cells, while chemokines that activate $CCR7^+$ mature DCs did not elicit antitumor activity, suggesting that immature dendritic cells are targeted by the DNA vaccines tested (Biragyn *et al.*, 2001). β-defensins were also injected into newborn piglets infected with *Bordetella pertussis* (Elahi *et al.*, 2006). This pathogen is responsible for acute respiratory tract infection in young children and infants worldwide (Crowcroft *et al.*, 2003). The development of a pertussis porcine model allowed investigation of the role of porcine β-defensin 1 (pBD-1) against respiratory infection with *B. pertussis*. pBD-1 has significant homology with human β-defensin 2, and its expression was observed in 4-week-old pigs resistant to *B. pertussis*, while newborn piglets developing severe bronchopneumonia did not express pBD-1. Interestingly, the injection of pBD-1, which displayed antimicrobial activity against *B. pertussis*, conferred protection against pertussis suggesting that the defensin was acting as a natural antibiotic or by modulating the innate immune response like human β-defensin 2 (Elahi *et al.*, 2006).

Defensins could be therefore good candidates for drug development. However, most of the defensins playing a role in immune response regulation tend to be induced in response to pathogen infections, suggesting that the use of probiotics to activate the induction of these endogeneous antibiotics could be another alternative for the treatment of infections. Probiotics are live microbes with beneficial effects on human health (Isolauri *et al.*, 2002), which act by interfering with pathogens (Reid *et al.*, 2001) and through the modulation of mucosal immunity including the production of immunoglobulin A and cytokines (Isolauri, 2001), suggesting that probiotics may prevent the invasion of commensal and pathogen microorganisms (Ouwehand *et al.*, 2002). Recently, the probiotic bacterium *E. coli* Nissle 1917, which is apathogenic, immunomodulatory and able to colonize the gut (Hockertz, 1991; Lodinova-Zadnikova & Sonnenborn, 1997), was shown to induce the production of human β-defensin 2 in Caco-2 intestinal epithelial cells in a time-and dose-dependent manner (Wehkamp *et al.*, 2004). This finding suggests that probiotics may stimulate innate defences to protect the epithelial barrier against commensal and pathogenic microorganisms.

In conclusion, the chicken genome has been revealed to code for a large family of avian β-defensins which are constitutively expressed at the mRNA level in different tissues. However, a novel AvBD, to date not annotated in the chicken genome, was also discovered and is specifically expressed in the spleen and skin. Despite the observation of slight up- and down-regulation of certain AvBDs at the mRNA level in response to systemic salmonellosis or to *S.* Typhimurium infection, suggesting a role for the AvBDs in the modulation of the immune defence, more studies to determine how AvBDs are regulated and their functions at the peptide level

remain to be carried out. Indeed, avian β-defensins might play an essential role in chicken immunity, as observed is for mammalian defensins, which are considered as innate antibiotics and immunomodulatory molecules. Therefore, a better knowledge of AvBD function may permit development of specific treatments against *Salmonella* infection.

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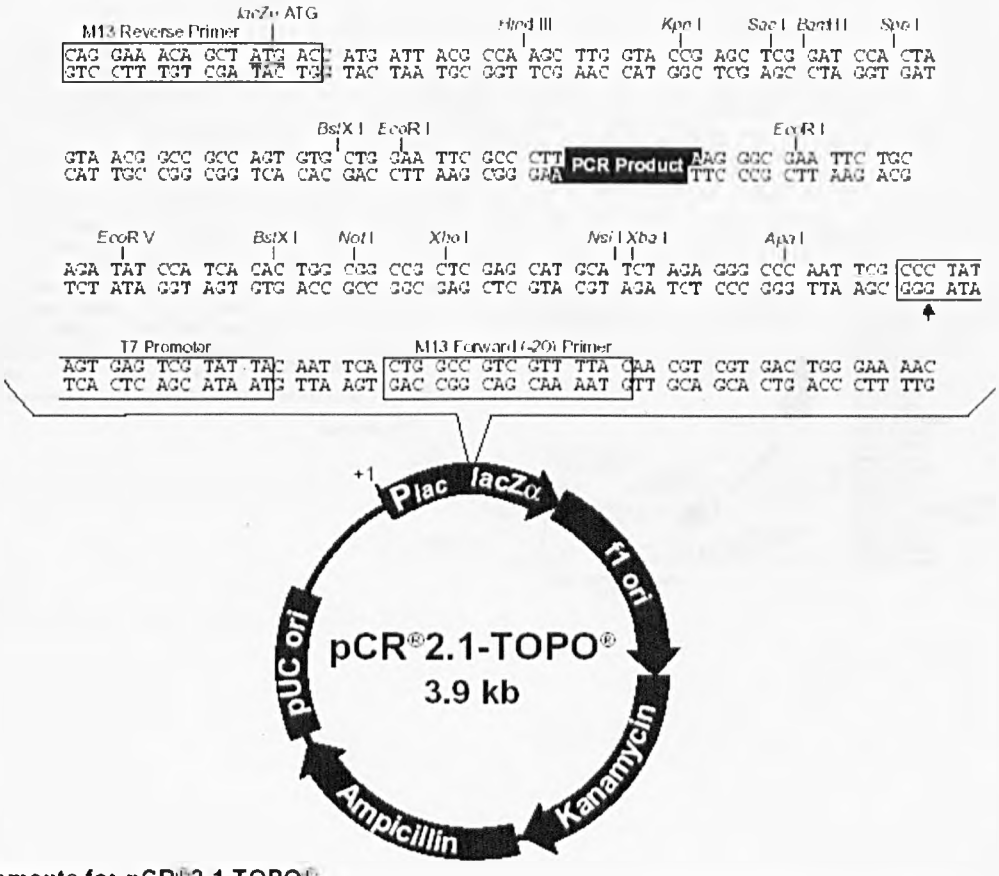
Appendices

Appendix 1: pCR2.1-TOPO vector map (Invitrogen).

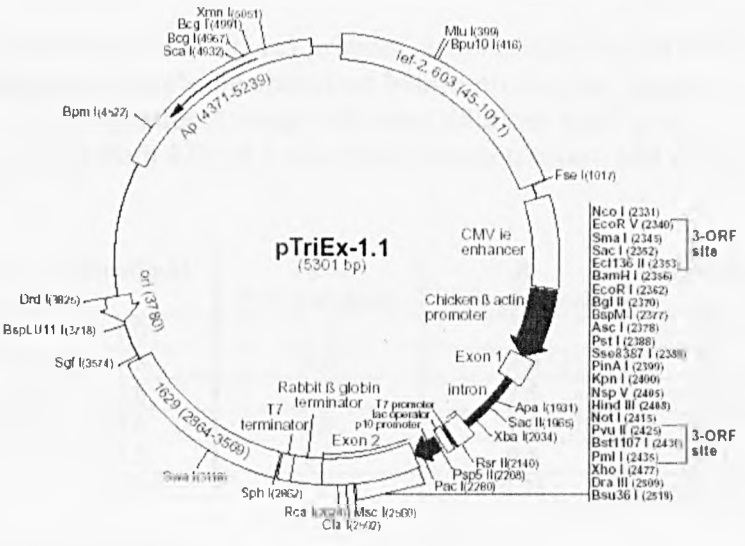


Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Appendix 2: pTriEx-1.1 vector map (Novagen).

pTriEx-1.1 sequence landmarks	
CMV ie enhancer region	1079–1143
Chicken actin promoter region	1149–1726
Vertebrate transcription start	1727
T7 promoter	2150–2166
T7 transcription start	2167
lac operator	2171–2191
p10 promoter region	2205–2318
p10 transcription start	2249–2250
Multiple cloning sites (*Nco* I–*Dra* III)	2331–2512
HSV•Tag® coding sequence	2111–2176
His•Tag® coding sequence	2183–2506
Rabbit globin terminator region	2561–2800
T7 terminator	2801–2851
pUC origin	3780
bla coding sequence	4371–5230



Appendix 3: Optimising primer concentrations for TaqMan assays.

1. Procedure:

Having designed a suitable pair of TaqMan primers, the concentration at which they are used in the TaqMan assay needs to be optimised before running an assay to quantify mRNA expression in test samples.A range of primer dilutions need to be prepared for optimisation, ranging from 1.0 – 0.1 μM (final concentration), and details are given in the table below.

[Primer]	Diln.	[μM]	[Final] μM	F (100 μM stock)	R (100 μM stock)	DEPC-H_2O
I	1:2	50	1.0	2.5	2.5	5.0
II	1:2.5	40	0.8	2.0	2.0	6.0
III	1:3.3	33	0.6	1.5	1.5	7.0
IV	1:5	20	0.4	1.0	1.0	8.0
V	1:10	10	0.2	0.5	0.5	9.0
VI	1:20	5	0.1	0.25	0.25	9.5

The 96-well plate was then filled as follow:

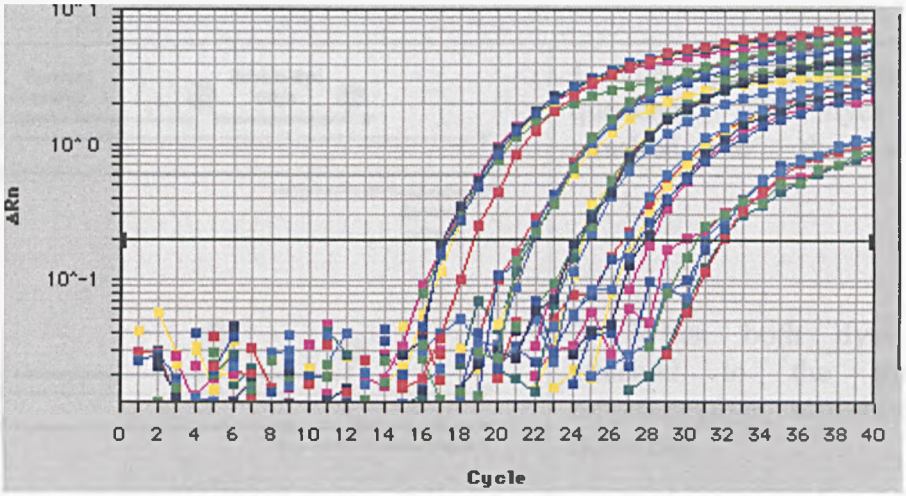
		1	2	3	4	5	6
Primer							
A	**I**	1:10	1:100	1:1000	$1:10^4$	$1:10^5$	NTC
B	**II**						
C	**III**						
D	**IV**						
E	**V**						
F	**VI**						
G							

2.5 μl DEPC-H_2O is added to the NTC wells. Then, 7.5 μl Master mix is added to each well, and 0.5 μl of each of the various primer dilutions to the appropriate wells (I to VI). Finally, 2.5 μl of each RNA standard is added to each well, as appropriate (10^{-1} to 10^{-6} in wells 1 to 5).

2. Example of result obtained:

The primer concentrations I & II (across the 5 dilutions of standards) were compared, and verified that they are almost identical. Then primer dilutions II & III and so on, were compared. When the shape of the curve begins to change at the exponential part of the curve (i.e. altering the Ct value), it indicates that the primer has become limiting and so the next highest primer concentration is optimal (see Figure).

Figure: AvBD 3 primers optimisation - probe at 0.1 µM; primers from 1 µM to 0.1 µM; RNA dilution from 10^{-1} to 10^{-6}.

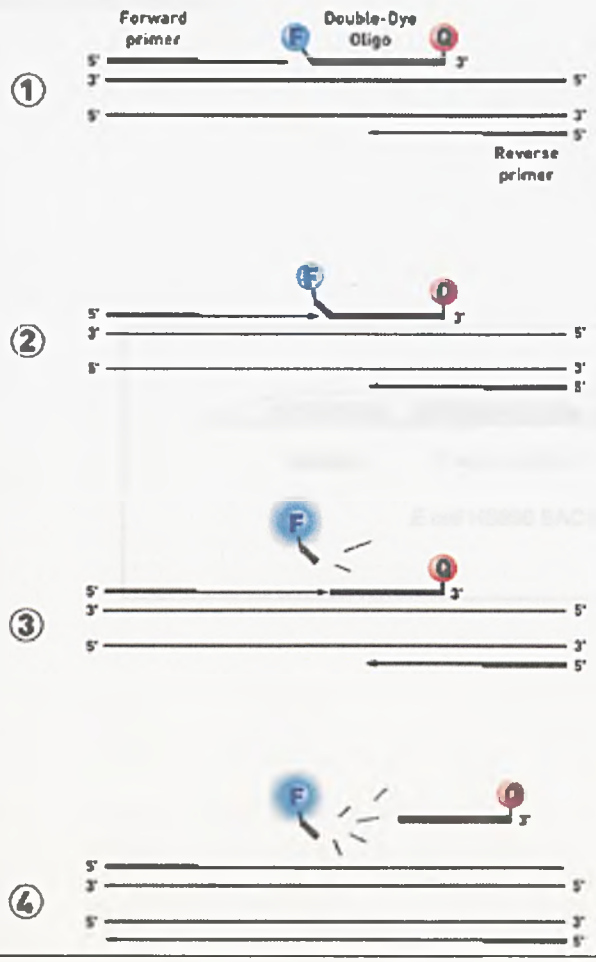


With a AvBD 3 probe concentration at 0.1 µM, the AvBD 3 primers concentration selected was 0.8 µM.

3. Results:

	Probe concentration µM	Primers concentration µM
AvBD 1	0.1	1
AvBD 2	0.1	1
AvBD 3	0.1	0.8
AvBD 4	0.1	0.8
AvBD 5	0.1	0.8
AvBD 14	0.1	0.6
28S	0.1	0.6

Appendix 4: Schematic diagram of real-time quantitative PCR (from Eurogentec/ EGT Group)



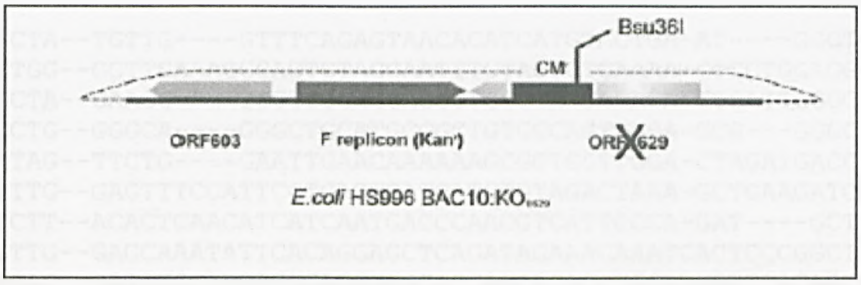
1- The reporter (R) and the quencher (Q) dyes are attached to the probe.

2- When both dyes are attached to the probes, reporter dye emission is quenched.

3- During extension, Taq/DNA polymerase, which also has a 5' exonuclease activity, cleaves the reporter dye from the probe.

4- Once separated from the quencher, the reporter dye emits its characteristic fluorescence.

Appendix 5: Bacmid, BAC10:KO$_{1629}$ map (Zhao *et al.*, 2003). Bacmid contains 1. the F replicon (Kan^{R}) from *E.coli* allowing the viral genome to be amplified in *E.coli*; 2. a chloramphinicol acetyl transferase cassette (CM) inserted in the ORF1629 involved either in nucleocapsid packaging or modification of the virion RNA polymerase; and 3. a single Bsu36I restriction site to provide a linear viral DNA. The knockout strategy of ORF1629 allows to not initiating virus infection unless a recombination with an appropriate transfection vector.



Appendix 6: Nucleotides pile-up to characterise possible promoter regions of the avian β-defensins. Alignments carried out with CLUSTAL W (1.83).

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AvBD6  ----------GTCTTGTTCTCTGAATACCATG-GCAGGCTGTGGGTGAAAGAT-TCAT-C 47
AvBD7  -----------GCTCATACTTTTATTGATATTCATGGGTCAAAGATAGCATTC-TGGTAC 48
AvBD3  ACCCATTCACATATTTGACTAACTCTTAAGTAAATAAGTTTGTCCTGATGTCC-CAAGCT 59
AvBD1  -------------TGTTGCTGTTTCT--GGCCACCAAGACCTCCCCGTCCACT-TGCTCC 44
AvBD4  ----------GAAATGTTTGTTGGCTGCAGAAGGAGATCTAGGAGGAAATTTG-TCTTGG 49
AvBD2  -------GATTGCTTTTAAATGTCTCTTGTCAGCTGGGAACAGATTTGGTTGT-TTTTGG 52
AvBD11 ---------------------------AGACAGTAAACACTGCAGCAGCACCT-TGGCCC 32
AvBD12 AATTTCTCAGAAGCCAGAGCTGGAATGGCCTTTTTGGCAGGAAAATCACAGCC-AGGTTT 59
AvBD9  ---------------GTATGCTTGCAGTGCCCCGTGGCTGGGAAGGGGACAGT-CTGTGA 44
AvBD10 -------------CATTGCTTTCCCTCAGTAAGAGGGATGGGGAGACAATGCTACAGGAC 47
AvBD14 -------GAGCCAAGCTACTCTCTCTGATTTTAGAAGGAGTGTATCTGGGATCTCTGTCT 53
AvBD13 ---------------AAACGTGTGCAGAGACTGGGAGCTGGAAATGAGAAAAATACGAAA 45
AvBD8  ----------TGATTTTGTGCTTATGGAAACATTGCCTATTAGTTATAAAAGAGACCTAG 50
AvBD5  --------CATTTTGCCAACCAACAATGGCTGACATACAGCGAATTCTTATGCAGAGTGG 52

AvBD6  AGCCTA--TGTTG----GTTTCAGAGTAACACATCATGTACTGA-AT----GGGT--GTC 94
AvBD7  AACTGG--GGTTGAAAGGGACTGTAGGAATTTCTAGTCTCAAAA-GTCCTGGAGG--GTC 103
AvBD3  GAGCTA--GAAGG----TCCTTCGAGGAGCCTGGGTAAGACAGA-GTGATTGGGC--AGG 110
AvBD1  TCCCTG--GGGCA----GGGCTGCATGGGGCTGTGGCAGTGAGA-GCG---GGGC--TGG 92
AvBD4  AAATAG--TTCTG----GAATTGAACAAAAAAGCGGTGGTTGGA-CTAGATGACC--TTC 100
AvBD2  AATTTG--GAGTTTCCATTCCTGAGTGATCATCTGTAGACTAAA-GCTGAAGATC--ATG 107
AvBD11 TCTCTT--ACACTCAACATCATCAATGACCCAACGTCATTCCCA-GAT----GCT--TTC 83
AvBD12 CAGTTG--GAGCAAATATTCACAGGAGCTCAGATAGAAACAAATCACTCCCGGCT--TGC 115
AvBD9  CAACAC--CATGTCCAAGAGCCACGGGGCATCAGCACACCTGCATGGTTTACAGT--ATT 100
AvBD10 TCATTA--GCAAGCAATAGGTCCAGGGACAATGCTGGGACTGGGCTCCAACAGCT--GAC 103
AvBD14 CCAGTACTGATTTTTACCAACTATGGAAGAACTTTGCATCCTTCATCCTTACATTTAAGC 113
AvBD13 CACTTTGATCCTGCAAACTCTTGGGTGGGTTTCCAACG-TCACATGCTGGAGTCT--ACA 102
AvBD8  GAAAAA---ATTGCTTAAATTATAAGAACTGGATACAATTTGAAGGATAACAATTAATTT 107
AvBD5  TGATGAAAGCTTGTCATTCATGGGGCTGAATGGAGATGTCATCCCGTGCGGCGTCTTCCC 112

AvBD6  AAAAA---GAGAATTCTTAGTGCAAGAA-GGCCA-AAACTCAAAAAATCAAAGATAGAAT 149
AvBD7  AGTGA---GGAGATGGTGAGGCCTGGTT-GCCTGCAGATACATTCAGGAACGTTTTGAAT 159
AvBD3  GTGGT---TTCAGCAGCCAGCTTTGAAC-TGCCTGCACTGGTAGGCAGTGCCAATTAAAC 166
AvBD1  GGAGT---TCTGTTATT--GCTGGGTGT-GACCATGAGTGG-ATGATGTGCCAGGATGTC 145
AvBD4  ATGGTCTTTCCAACCTTAATGATTCCTT-CTCTACTCTTGCTTTAGAAAGGTTTGAGAAC 159
AvBD2  AGCAAATTGCTTTTGCTAAGTTTCAA---CACTGCGAAATGAACACGTTATTTCTGTCAT 164
AvBD11 TCCCCCACCAAAACAGT-GGCTTATTTT-CTCCACGTGGCCCATTAGCAATTAATAAA-- 139
AvBD12 AGCAGCTCCAGAGACTTTCTTTGCAGGT-TGCCACCGCTCAGCCCATCATCTCTCCGGGC 174
AvBD9 ATGGATCACA GGAACCCACCTCCCTCCT-GCTCCAATCCTGTGTCCTCTCTGGGTGCAGC 159
AvBD10 TCCCAAGTCCTATGCATGTTCCATGCCC-GTGCCCATGCTGGAACAAATCTGCA-ATAGC 161
AvBD14 AGCCCCTGAATGAAAAAAGGGGTGCATG-GCCTACAGCCTATTTTTCCTCTTGGTGTGCT 172
AvBD13 AACGCA--CCAACAGCCAGGCTGGGTCA-GCCCCCACATCCTGTCTCCAGCAGCAAACAG 159
AvBD8  TTATTTTACTTTTAATTTATTTTGTTAT-AATTCAAAAATATAAATAAAATATCTGAAGT 166
AvBD5  AGCAGCTCTCAAGTGAAGCATAGAGAGCACGCTTCACTGGAGGAGAGGAACACAGAGCTC 172
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AvBD6  -TTGGGAACAATAGGGGA-TGAA-----AGGGACTGTAGGAGCTTCTAGTCTTGAAAGTC 202
AvBD7  ATCTGCAACTTCACTCAAGTTCA-----TTGTATCACATCAGCTTTTGGTTTGGGTCATT 214
AvBD3  CTTCATACAGTGTGGCAGCTCCAGGTACAAGCATTTCCCTGACTCTCAAAGCCCAGATCC 226
AvBD1  TTTTGTGAGCCTGGGGAAACGGAGGTGTTTGGGTGGAGTGG--TTTGGGTGGCCAG--CT 201
AvBD4  GTGCTTCGTGTTGCCTTGCATGAATGTAGTAAGAATTGTCATGTTCTGGTTTCTGAA--- 216
AvBD2  TTATAAGCTCGGATGTAGAACCACCTCACTGTGCTCAGTGGGGTTTATTTATTAGATAAA 224
AvBD11 -TTGAAGTTAACAAGTAA--CGAAGCCCTTGGGGTGGGTCGGTTTTGAGAAGCAGCCTTC 196
AvBD12 ACTTTGCTGGTGGAGGACGAAGAAACCCCTGCACCTCATTTTTCCTGGCAACCCACCCAC 234
AvBD9  CCATCAGCTGTCACAGGGCATGTGAGCACAGGGTGTCATTTGGTCCTTGTTCAGGT---C 216
AvBD10 CAGAGGTTTGGGGCTTGGCTGATAGCCCCATTATACAACTGGCCGTCACGGTCAGT---- 217
AvBD14 AAATAGCCATTCACATCTCCAGTGAA-GCAAAATATAGACAGTGGTGAAATCACCTCTGC 231
AvBD13 GAGCAGACTCCCCAGTGCTTGTTGCGGTTTGTTGACAGAAACCTGCTGGTATTTTTTCCT 219
AvBD8  CCATGGGATTCAAATGCA--GGAGTCTTCAGTGCAGATGATACTGTTTGTTTTTGCATGT 224
AvBD5  CTTTGTGCTGGGAGAGAGGGTTGCGTGCCAAGGCTAACGGATGGGGATGAAGTGTGTCCC 232


AvBD6  TTAGAAGCTCAAAGAAGAGGAGATGGTGAGGCCTGGTTGCCTGCAGATACATTCAAGGAC 262
AvBD7  CGGAGCGTTGGGTCAAAGAGAGATGATTGGGCCTTGTTGCCTGGAGTCACATTCAGGGAC 274
AvBD3  TTCGTTGTCTCTAGTCTGGTGATGGGCTTTGCATGAGTGTTGGCACGAGCATCTCCTGAT 286
AvBD1  TTGCATGGATGTGTAGCACCAACTAACCCTACATG-----TAGTGTG-GCATCTCCAGGT 255
AvBD4  TTAACAACTTCCACAGAGCAGGTTCAATTTATTGCAGTGTATTGCAACATGTCAAGCAAT 276
AvBD2  ACGTTGAACCGGGCATGAGGTGTTGTCTGTATTTTG--GCCAAGGAGTATTTGCAAAGCG 282
AvBD11 CTGGAGGCATGGTGAGGAGGTATGGTTACCTCTCTGCTGGCTTTTGTGACCCTCTGTCAT 256
AvBD12 CCAAAATACCAGCTGCCAGGTAGAGGAAGCA---------AAAGTGAAGCAAACCAGCAT 285
AvBD9  AGGCATCTTCAAATGTGTTGGGTTGCAACATCTTCATACATCACCAATCTCTACAGTCAT 276
AvBD10 ---GGAAATTCACTGTTGCAGATTGCAACATTTTCACCAGACATTTCACTGCAGCCCGCG 274
AvBD14 ACATGGCATGGCATGGGCTGGCACAGGATGGCATAG-TGGCATGGGGTGTCTGCCCCATT 290
AvBD13 TTTTTTTCTTTCTTTTTTGCTTTTTTTTTTTTTTTGCTTTTTTTTTTTTTCCTCCACCCTG 279
AvBD8  AATGGAGAGAAGATCACTATATTTTGAATTTTATAG--ATATAAAACACATTTTCTTGAT 282
AvBD5  AAGAAGCGTCCAGCAAACCACAGCGCTGTGTTTCTCTTTCGCAAGGCAGGATGACACAAG 292


AvBD6  TATTTGAATATCTGCAACTTCACTCAAGTTG-ATTGTATCACACTGGATTTTGG-TGTGG 320
AvBD7  AATTTGAATATCTGCAACCTCATGTGAGTTC-ATTGAATTGTATAAGATTTTGG-TGTGG 332
AvBD3  -GTTCAGATATCCTCTACTGTGCTTGAAAT--ACTATTTTACTTCATCCCTTAATTGTGA 343
AvBD1  -ACAAACATATCCGTGACT--TCCAGAGCC--TGGATCTTCTATTGATCCTCG--TGTAA 308
AvBD4  --TTAGGATAAACGTCTACTTAATTAACATG-GTAATTAAAAGTTGGATTTAAAATG-GC 332
AvBD2  TGGCAGGAAATCTGA------ATTAGAACAG-CTTAATAAACACAAATCCTGAGATATGT 335
AvBD11 TGTTCGGGCTTAGCC------GTGTGCAGCG-GTCAAGCTGTACTCGTTGCTC--CGTGG 307
AvBD12 TGTTTGGAGCTGCGGCGCTGGGTCAGAACGACACAGAGGATTGCTGAGCGCTGAATGTCA 345
AvBD9  AGGGCAAAGAACTCTCACCACTCCTCCTCCCCTGAAGTGTCTGCACTGTCCAGACCCACA 336
AvBD10 ATATGGGGGATAACCAATTGCTTGACAAAAG-GTTGGGTGCAGTTACTTATTGACAGGGC 333
AvBD14 TTTGCAGGCTTATTGAGGTGGGGCATGTTGTGCTATGCATCCACACAGGAATTGTAATTA 350
AvBD13 CACATGAGGTGTCCGA-----GAGCGGGCAGTTCCCAGAGCAACTGTTTGCTG-TCATGT 333
AvBD8  CATATGAAATGTGCATCCCTTAAAAAAGTGATATTTCAATAGGCAGTTTCTAGAAAATTT 342
AvBD5  AGCCGGGGCTGAAGGCCTAGGGGGAAAGCCATTCCGTGTCATCTCTGACAGGGGAAGAAA 352
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AvBD6  GTCA-TTTGAGGCATGTTAGAAGCAGTTGCTCAACTCTCAG---TCGG-----GAGATAA 371
AvBD7  GTCA-TTTGAGGCATGTTATAAGCAGTTGCTCAATTCTCAG---TCGG-----GAGATAA 383
AvBD3  TCAACTTTGGGGACAGCCAGTTGTGAGGTCATAGAATTTTAGTCCTGGAAATTGGAGAAA 403
AvBD1  CTG--TTGGAATTGCGTTGGCTGTTGGCCCA-AGCACCT----CCTGA----TGTGTCAA 357
AvBD4  ATAGTTCTGCACAGAAGCTTCTGCCATGATCTTACGTGTGGTCCAGGGAACCCTTAAGAC 392
AvBD2  ATT--TATATCCCATGCTGCTCCTTGCTTTCTTTCCCCCAGA--AATGCCCACAGAGCAT 391
AvBD11 CCTGCCTTCCACATACCCTACTGCCAAAATCCCCTGGACAG--CCTGG--TCTGTTCCCA 363
AvBD12 CCG-----GGATATAATTAACTCCTGCCTGCTCCCTCCCTCCTCCAGG-----ATGCCAT 395
AvBD9  GCCTTTATAAGTGCAGGGACCAGCCATCTTCTGCCTCATACA--TCAG---CTTCTGAAC 391
AvBD10 ACTCAGCCGTGAAACTTTCACAGTC-CCACAACCCCTATAAATGCCAG-----GCGCCTT 387
AvBD14 GAGGTTACAAGACACGTCCTTCAAAGCTGTTATTTATAAGATTGCTAAATCCCTGGTGAT 410
AvBD13 TGAAATGCACCTGAGATTGACAAAGTTCCCTTCGCTCTGGGAGCGTTA---CACAACTCC 390
AvBD8  ACTCTGTTGCATAAAAGCTAACTGCAATTTGCAAGTTGTAGGTTGTAA-------GTCAT 395
AvBD5  CAGG---AAAAGGTGCTTTGGGAACAATCGGTGGTGTCAGGGATACTGCCTGCGTGGCAG 409


AvBD6  CCATTCTGTGCCTTTTGGTCTTGTTGTGTCTGTCC-ATTGCAGATAAGGATTTCA----- 425
AvBD7  CCATTCTGTGCCTTTTGATCTTGTTGTGTCTGTCC-ATTGCAGATAAGGATTTCA----- 437
AvBD3  TTGTTCCTGGAAATGTTCCCAGGTTCTG-CATGGT-AGTGCACAAAATCATATCACTACC 461
AvBD1  CTCTGCTGTGACATTTGATTAAGTAGAGACAGAAG-AGAGAAAACAAATATACCAGTGGT 416
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AvBD12 CCCGCCTGCCAACGCCATGCAGAGGCTT-CTGCACAATCTCA--CGCTCAGCCCTGCTGC 452
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AvBD14 CACATTCATCAAAGCTTTATAAAGAGAGGCTCATTCCTTCCTCTTGGTCTCAGCAGCTTC 470
AvBD13 CAGCCCTATAAATCCAGGATTTCCCTCTTCTCTAT--CTCCCTACAGCCCTTCTGGTGGT 448
AvBD8  ACATGATGTTTGGGTCAA--TAGTCCATACAGAATAAATGCA--AGATTTTTTTGGTGGC 451
AvBD5  GAGG-ACGCCAGCTGGGATCAAACTGCTGCTGCCAGCAAGAAAGGAACCTGCCCTGTTTT 468


AvBD6  -------------CATCCCATCCGTGGCCATGAGGATCCTTTA-CCTGCTGCTGT-CTGT 470
AvBD7  -------------CATCCCATCCGTGGCCATGAGGATCCTTTA-CCTGCTGCTGT-CTGT 482
AvBD3  C---CCAGGCTTCTAAGCTGTTTCTGTTCTGCCATGTCCTT---GTTGCTTTTGT-TCAT 514
AvBD1  A---CTT--CTGACACGTTGTCTGTGCTAGAAAGTGTATCTTGTGTGGCCTTGGT-TTCT 470
AvBD4  T---CAC--TCCTCAGCCCA-CTGTGTCTGTAGGTGGACAACATCTCAGTGTCGT-TT-- 503
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AvBD12 T---CCC------CAGCAGGACCAAAGCAATGAGGAACCTTTGTTTCGTGTTCATCTTCA 503
AvBD9  C---CTTTTCTTCCTTGTTG-CTGTTCTCTTCTTCCTCTTCCAGGCTGC---------- 496
AvBD10 C---AGTCCACAACTGAGCCATGAAGATCCTCTGCCTGCTCTTCGCTGT---------- 493
AvBD14 AGGGCGACACGACAATGTCAACCAAAGCCATGGGCATATTCCTCCTGTTTCTTGTTCTCC 530
AvBD13 GGGACGCCCACCCACATTCAGCCATGAGGATCCTCCAGCTGCTCTTTGCCATCGTTGTCA 508
AvBD8  AATTTTTTTTCCCCCTAGTGGCTGTTGTGTTTTGTGACACTGAATTTGGACATGAAGATC 511
AvBD5  TTCTTCTCCCCACAGCTGTGACCCTCCGGGCATCTCCCAGCCATGCAGATCCTGA-CTCT 527


AvBD6  CCTCTTTGTGGTGCTCCAGGGT---------------------------------------- 492
AvBD7  CCTCTTTGTGGTGC------------------------------------------------ 496
AvBD3  CCACTCTGCAGCCTCGTGAGGAACCTGCTCCAGGCATCAGCCATGAAGATCCTGTACCTG 574
AvBD1  CCCCTCTGTAGCCCTGTGAA-AACCCGGGACAGACGTAAACCATGCGGATCGTGTACCTG 529
AvBD4  ---CTCTGCAGTG--ACAGGATTTCCCAGTCTGCCTTCTGCCATGAAAATCCTTTGCTTT 558
AvBD2  TCTTCCTTTTCCC---TGTAGCAGCTCAGCAGATCTGCAGCCATGAGGATTCTTTACCTG 562
AvBD11 CCTC------------------------------------------------------- 474
AvBD12 TCTCCCTGCTCGCTCACGGTAAGGCTGGGGGTGGCAA---------------------- 540
AvBD9  ------------------------------------------------------------
AvBD10 ------------------------------------------------------------
AvBD14 TGGCAGTACC------------------------------------------------- 540
AvBD13 TTCTCCTCCTCCAGGATGCGCCTGGTAAGGAC-------------------------- 540
AvBD8  CTTTACTTTCTCTTGGCCGTTCTCCTCAC---------------------------- 540
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AvBD5  CCTCTTTGCTGTC---------------------------------------------- 540


AvBD6           ----------------------------------------
AvBD7           ----------------------------------------
AvBD3           CTCATCCCCTTCTTCCTCTTGTTTCT---------------- 600
AvBD1           CTCCTCCCCTT------------------------------- 540
AvBD4           TTCATCGTGCTCCTCTTTGTGGCAGTTCATGGAGCTGTGGGT 600
AvBD2           CTTTTCTCTCTCCTCTTCCTGGCACTCCAGGCTTCTCC---- 600
AvBD11          ----------------------------------------
AvBD12          ----------------------------------------
AvBD9           ----------------------------------------
AvBD10          ----------------------------------------
AvBD14          ----------------------------------------
AvBD13          ----------------------------------------
AvBD8           ----------------------------------------
AvBD5           ----------------------------------------
```