ANALYSIS OF INNATE IMMUNITY RELATED GENES IN DIVERGENT GERMPLASMS OF CHICKEN

Dissertation

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By

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ABSTRACT

Although chicken strains show differences in susceptibility to a number of diseases the underlying immunological basis is yet to be elucidated. Avian heterophils have been reported to express toll-like receptor and beta-defensin genes. In the present study heterophils were subjected to LPS stimulation and total RNA extraction. Differential gene expression was studied in broiler, layer and indigenous Aseel strain by Real Time RT-PCR SYBR Green chemistry. The expression of the 14 AvBDs, chTLR 1, 2, 3, 4, 5, 7, 15 and 21 was detectable in heterophils. The expression level of most of the AvBDs and chTLR4 significantly increased ($P<0.05$) 3 hours post *in vitro* lipopolysaccharide challenge. Higher expression level and stronger activation of chTLR4, most AvBDs, NFkB-1 and IRF-3, in heterophils was observed, with the stimulation of LPS in layer compared to broiler, and in Aseel compared to both layer and broiler. The expression level of most of the AvBDs and chTLR4 significantly increased ($P<0.05$) 3 hours post *in vitro* lipopolysaccharide challenge. This investigation will allow more refined interpretation of immuno-genetic basis of the variable disease resistance/susceptibility in divergent stock of chicken including indigenous breed.

Keywords: defensin, toll-like receptor, lipopolysaccharide, heterophils, innate-immunity.

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Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

%	Per cent
µg	Microgram
µl	Microliter
°C	Degree Celsius
EDTA	Ethylene diamine tetra acetic acid
e.g.	exampli gratia
et al.	*et alibi* (and others)
etc	*et cet-er-a*
g	grams
i.e.	id est (that is)
mg	milligram
ml	milliliter
bp	Base pair
dNTPs	Deoxynucleoside triphosphate
M	Molar
Min	Minutes
mM	millimolar
ng	nanograms
nm	nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
nmole	nanomole

SDS	Sodium dodecyl sulphate
sec	Seconds
TBE	Tri borate EDTA buffer
TE	Tris-EDTA buffer
UV	Ultra Violet
V	volts
viz.	Videlicet (namely)
cDNA	Complementary DNA
rpm	Revolutions Per Minute
AMP	Antimicrobial Peptide
AvBD	Avian-beta-Defensin
Ct	Threshold Cycles
chTLR	chicken toll-like receptor
hBD	Human Beta-Defensins
IRF	Interferon Regulatory Factor
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide
NFκB	Nuclear Factor Kappa B
PAMP	Pathogen-Associated Molecular Patterns
PRRs	Pattern Recognition Receptor
qRT-PCR	Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction
TF	Transcription Factor
TFBS	Transcription Factor Binding Site

CHAPTER – I

Introduction

Immune response is the main mechanism of defense employed by the animals against foreign invasion and aids in restoration of homeostasis (Abbas and Litchtman 2003). Host defense against invading microbial pathogens is elicited by the immune system which consists of two components: innate immunity and adaptive immunity. Both these components of immunity recognize invading microorganisms as non- self which triggers immune responses to eliminate them (Takeda *et al* 2005).

Before the evolution of adaptive immunity in higher vertebrates added complexity, specificity, and memory to fight microbial challenge, a simpler, non-specific ancient system of innate immunity evolved 2.6 billion years ago and continues to function as the principal defense for almost all living organisms (Kaufmann 2004). Innate immunity is necessarily rapid, redundant, and multifunctional (Ganz 2003).

The importance of the innate immune response is its immediate, nonspecific, defensive effects on a broad range of pathogens before the establishment of a more specific adaptive immunity, which usually takes several days. It works through non-rearranging receptors called pattern recognition receptors (PRRs) which include Toll like receptors (TLRs), scavenger receptors, and nucleotide binding oligomerization domains containing proteins (NODs). PRRs recognize conserved microbial signature molecules which are class-specific and mutation resistant, collectively called as pathogen associated molecular

patterns (PAMPS) (Janeway and Medzhitov 2002). The Toll-like receptor (TLR) family is a highly conserved group of proteins that participate in pathogen detection and in the initiation and regulation of innate and adaptive immune. To date, 10 TLRs have been identified in chickens (TLR1 (types 1 and 2), TLR2 (types 1 and 2), TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21 (Boyd *et al* 2001, Fukui *et al* 2001, Higgs *et al* 2006, Iqbal *et al* 2005, Kaiser 2007, Keestra *et al* 2007, Philbin *et al* 2005, Roach *et al* 2005 and Yilmaz *et al* 2005). The function of TLRs is related to their ability to recognize conserved chemical structures called pathogen-associated molecular patterns (PAMPs). Numerous bacterial PAMPs have been defined, and their respective TLRs have been identified. The most extensively studied TLR is TLR4, which recognizes LPS, a critical component of Gram-negative bacteria. TLR2 is involved in recognition of a variety of microbial components including lipopeptides from Gram-positive bacteria and zymosan from fungi (Takeda and Akira 2005). Unmethylated CpG motifs of bacterial DNA are recognized by TLR9 in mammals and by an unknown receptor in the chicken. Bacterial flagellin is recognized by TLR5, which is found in both mammals and chickens.

The other important innate immunity effector molecules are defensins, a family of small cationic peptides with broad spectrum antimicrobial activity against bacteria, fungi, protozoa and enveloped viruses (Zasloff 2002) via multiple mechanisms, such as pore formation and membrane disruption (Evans 1995). They are induced in response to challenge by lipopolysaccharide, by a regulatory pathway similar to that used by the mammalian immune system, involving toll-like

receptor (Hancock and Scott 2000). Based on the arrangement of cystein residues, these peptides are further grouped into three subfamilies, namely α-, β-, and θ-defensins (Ganz 2003). Chickens produce only β-defensins, previously known as gallinacins, with 14 avian beta-defensin (AvBD) genes being discovered (Harwig 1995, Lynn 2003 and 2007 and Xiao 2004).

The evolutionary strategy of the innate immune system has been to recognize a few highly conserved, constitutive structures such as lipopolysaccharide (LPS) or peptidoglycan (PDG), and these structures are present only in foreign microorganisms (Medzhitov 2001), which rapidly limits the expansion of invading pathogens and provides time for more effective host adaptive immunity to be generated.

Heterophils are important mediators of innate resistance in poultry. They are the dominate granulated leukocyte in the acute inflammatory response capable of a broad spectrum of antimicrobial activity. What makes them more important is that avian heterophil lacks myeloperoxidase and depends primarily on nonoxidative mechanisms for antimicrobial activity. Heterophils express high level of anti-microbial activity from recognition of PAMPs to killing the foreign microbes.

The LPS is a gram-negative bacterial cell wall component, which mimics the effects of a bacterial infection (Leshchinsky and Klasing 2001), and has been found to be a very potent stimulus in immune response and stimulated the highest production levels of nitric oxide (NO) in chicken monocytes among several common PAMPs (He *et al* 2006). Also, LPS induced highest expression of the

kinases (p38 and ERK1/2) among several TLRs agonists including LPS, PAM, FLG, PGN, LOX and poly I:C (Kogut *et al* 2005).

The main focus of research so far has been confined to acquired immunity where mechanisms for antigen recognition, diversity, clonality and memory have been well characterized but the innate immune system has not been well studied. With the advent of the genomics era, great efforts have been made by researchers to identify and characterize the primary gene components of innate immunity including chemokines, cytokines, complement factors, and toll-like receptors

The chicken strains/breeds show differences in susceptibility to a number of diseases (Zekarias *et al* 2002) and indigenous breeds of chickens are considered to be more disease resistant than their commercial counterparts (Dhinkar *et al* 2009). Intense and long-term selection for increased egg production and weight gain have been implicated in the immunological ability of modern improved stocks. Instead of identifying birds resistant to a single pathogen, it would be better to identify immuno-genetic indicators that would reveal which line(s) has the potential to mount the most effective immune response against multiple microorganisms.

In the present study differential expression of innate immunity related, toll-like receptor genes and beta- defensin genes have been analysed for constitutive and LPS induced expression in commercial broiler, commercial layer and in indigenous Aseel breed of chicken using quantitative real-time PCR. Such an investigation would help to better understand the genetic basis of the variable disease resistance/susceptibility observed in divergent stock of chicken including

indigenous breed which are thought to be hardy.

The specific objective of the study were:

1. Investigation of differential expression of Toll-like receptor genes (TLR 2, 4, 5 and 7) in divergent chicken germplasms.
2. Expression profiling of beta-defensins in divergent chicken germplasms.
3. Transcriptional profiling of TLR4 and beta-defensins in chicken heterophils before and after *in vitro* lipopolysaccharide (LPS) treatment.

CHAPTER – II

Review of Literature

The literature reviewed for the present study has been presented under following main headings:

2.1: Innate immunity and its protective role.

2.2: Toll- like receptors

2.3: Antimicrobial peptides (AMPs) and avian defensins

2.4: Regulatory and signaling pathway of innate immunity

2.5: Heterophils in birds.

2.6: Lipopolysaccharide (LPS) stimulation of cells.

2.7: Quantitative gene expression studies related to innate immunity in chicken

2.1: Innate immunity and its protective role

Animals are constantly exposed to millions of potential pathogens through contact, ingestion, and inhalation. The immune system can be divided into the innate and adaptive systems. Both systems are complementary and highly interrelated in a host's defense system. Innate immunity is an ancient and universal mechanism utilized by many organisms. In contrast, the adaptive immune system is an evolutionarily newer system, and induces a delayed antigen-specific immune response, which increases with successive exposure to the same microbes (Abbas and Lichtman 2003).

The importance of the innate immune system is its immediate defensive effects on a broad range of pathogens before the establishment of a more specific adaptive immunity, which usually takes several days. Host's ability to avoid infection depends on

their mechanisms of innate immunity (Hancock and Scott 2000). The innate immune system uses at least two distinct strategies of immune recognition: recognition of microbial non-self and missing self. The first is based on molecular structures that are unique to microorganisms and are not produced by the host. This directly leads to the activation of the immune response. The second is based on molecular structures expressed only on normal, uninfected cells of the host (Medzhitov 2003). Innate immunity provides an ever-present or rapidly inducible defense against microbial infection. The innate immune system is an evolutionary conserved system of defense that responds very rapidly in the early phase of the immune response. This naturally occurring first line of defense confers non-specific protection without previous exposure or memory against a large number of pathogens (Diamond *et al* 2000 and Froy 2005).

The recognition of foreign bacteria is the first and critical step in immune response. The evolutionary strategy of the innate immune system has been to recognize a few highly conserved, constitutive structures such as lipopolysaccharide (LPS) or peptidoglycan (PDG), and these structures are present only in foreign microorganisms (Medzhitov 2001). Therefore, the innate immune system does not need much flexibility to detect microorganisms. The molecular characteristics of these microbial components are named as pathogen-associated molecular patterns (PAMPs), and the host receptors that recognize these patterns are called pattern recognition receptors (PRRs). These recognize conserved microbial molecular patterns known as pathogen/ microbial associated molecular patterns (PAMPs/ MAMPs) (Hoffman *et al* 1999). PRRs have evolved to take advantage of three salient PAMP qualities: Firstly that constitutive expression which allows the host to detect the pathogen regardless of its life cycle stage.

Secondly the lass specificity that allows the host to distinguish between pathogens and thereby tailor its response and lastly that the mutation resistance which enables the host to recognize the pathogen regardless of its particular strain (Medzhitov 2001 and Gordon 2005).

These receptors can be either secreted or bound to host cell membranes. Most secreted-PRRs are plasma proteins such as mannan-binding lectin (MBL) and C-reactive protein (CRP), which can mediate antimicrobial effects by activating the complement system, assisting phagocytosis, or facilitating the binding of surface PRRs to foreign microorganisms (Schwalbe *et al* 1992, Matsushita and Fujita 1995 and Underhill and Ozinsky 2002). Several types of PRRs like complement, glucan, mannose, scavenger and Toll- like receptors, each with specific PAMP ligands, expression patterns, signaling pathways and anti- pathogen responses (Gordon 2005). Membrane-bound PRRs, such as Fc-receptors, complement-receptors and Toll-like receptors, play critical roles in phagocytosis, as well as activation of inflammatory signaling transduction pathways (Underhill and Ozinsky 2002). Because of the important roles of innate immunity, pathogens must overcome these defense mechanisms in order to establish their infections. Many pathogens evolve to possess virulence factors to evade innate immunity (Hackett 2003).

These evasion mechanisms indicate that innate immunity is important for host to resist infections. Evidence shows that stimulating the innate immune system provides both prophylactic and post-exposure protection. A sterile inflammation induced by a subcutaneous injection of casein, 24 hrs before disease challenges protected mice from lethal infection of gram-positive or negative bacteria. CpG-ODN injection protected

rodents and non-human primates against bacterial, viral, fungal, and parasitic infections (Klinman 2004).

Muzio and Mantovani (2001) studied that several microbial molecules or PAMPs such as lipoproteins and lipopolysaccharides, and whole live or killed organisms have been found to bind specifically and / or activate TLRs as opportunistic ligands.

In poultry, a Newcastle disease virus vaccine induced nonspecific immunity against subsequent infection with pathogenic *Escherichia coli* (Huang and Matsumoto 2000). The protection effects were suppressed with corticosterone and could not be induced by secondary vaccination. Subcutaneous and intramuscular injection of CpG-ODN in broilers 3 days before challenge with a virulent strain of *Escherichia coli* could reduce the mortality significantly ($P < 0.0001$) (Gomis *et al* 2003). These results indicate that innate immune stimulation protects hosts from infectious disease.

2.2: Toll-like receptors

During the evolution, multi-cellular organisms developed various mechanisms to discriminate between self and non-self, and efficiently protected themselves from the invasion by infectious pathogens. Innate immunity effectively recognizing and interacting with foreign products mainly depends on host germ-line encoded receptors. Inflammation is the response of tissues to invading microorganisms or tissue damage. This involves the activation and directed migration of many different cells, especially macrophages, from the bloodstream to sites of invasion. A cell such as a macrophage thus uses its TLRs to identify the presence of an invader and respond appropriately (Tizard 2004).

Toll receptors or Toll-like receptors (TLRs) are groups of important membrane

PRRs specifically recognizing different pathogen associated molecular patterns on foreign microbes (Akira *et al* 2001).

As early as the 1980's some people found that, in early *Drosophila* larvae, there was a transmembrane receptor that played a critical role in determining the embryo's dorsal-ventral polarity (Anderson *et al* 1985, Hashimoto *et al* 1988), and later this receptor was named as Toll receptor (Stein *et al* 1991). This receptor was also found to play a significant role in other physiological functions including antifungal activities (Lemaitre *et al* 1996).

Gay and Keith (1991) found that 18-Wheeler like Toll is a type 1 transmembrane receptor with an extracellular domain containing LRRs and a cytoplasmic domain intriguingly similar to the cytoplasmic domain of the human interleukin -1 receptor(IL-1R). Further Williams *et al* (1997) concluded that 18-Wheeler also plays a role in innate immune response of *Drosophila* through a similar signaling pathway and belongs to the same gene family as Toll.

The TLRs are homologous membrane proteins found in vertebrates, and these receptors are widely expressed in various mammals, birds and fish (Medzhitov *et al* 1997, Fukui *et al* 2001 and Oshiumi *et al* 2003). The first vertebrate TLR was identified in humans one year after the discovery of Toll receptor in *Drosophila*. This human TLR played an important role in the inducible expression of pro-inflammatory cytokines (IL-1 and IL-6) by activating NF-kB. Also, this TLR was critical in activating the naive T cell of adaptive immunity. Close investigation revealed that this mammalian TLR contained similar structures to that of *Drosophila*. In mammals, especially in humans, TLRs have been widely and intensively studied in recent years.

The Toll or Toll-like receptor family shares a similar structure containing leucine-rich repeats (LRRs) in the extra-cellular region, and a Toll/Interleukin-1 receptor (TIR) homology domain in intra-cytoplasma. Based on amino acid sequence and genomic structure, these mammalian TLRs could be divided into five subfamilies: TLR 2, -3, -4, -5, and -9 (Takeda *et al* 2003). The TLR-2 subfamily contains TLR-1, -2, -6, and -10, whereas the TLR-9 subfamily includes TLR-7, -8, and -9. The cytoplasmic domains of these TLRs are relatively conserved, whereas the extra-cellular parts vary among different TLRs, which confer specific binding abilities to different compounds or chemicals of foreign pathogens. The immuno-staining method was utilized to locate the exact positions of these TLRs on the host cells. Using specific TLR antibodies, the positive staining signals revealed that hTLR-1, -2, -4, -5 and -6 are preferentially expressed on the plasma membrane, whereas TLR-3, -7, -8, and -9 are usually localized to intra-cellular compartments (Matsumoto *et al* 2003 and Takeda and Akira 2005). These positional differential expressions of TLRs are consistent with the specific ligands binding abilities among them. Generally, the surface-expressed TLRs mainly respond to the cell wall components of foreign pathogens, whereas the intracellular membrane-expressed TLRs recognize nucleic acids such as RNA or DNA (Dunne and O'Neill 2005). In detail, TLR-1, -2 and -6 specifically respond to various bacterial components including lipopeptide or peptidoglycan from gram-positive bacteria, TLR-3 recognizes double-stranded RNA (dsRNA) from viruses during their replication, TLR-4 mainly recognizes LPS from gram-negative bacteria, TLR-5 responds to bacterial flagellin, TLR-7 and -8 can recognize single-stranded RNA (ssRNA) as well as imidazoquinolines, and TLR-9 responds to un-methylated CpG DNA motif from bacterium or virus and

hemozoin from malaria (Iqbal *et al* 2005 and Kawai and Akira 2006). The exact ligands for TLR-10 are still unknown, but TLR-10 shares a similar structure with TLR-1 and -6, and can heterodimerize with TLR-1 or -2 (Hasan *et al* 2005). The mouse TLR-11 was thought to recognize profilin-like ligands from some parasites (Yarovinsky *et al* 2005).

The specific repertoire of TLRs can be further extended by the heterodimerization or homodimerization capability of these receptors. For example, the heterodimser of TLR-2 and TLR-1 can specifically recognize bacterial lipopeptides (Wyllie *et al* 2000), whereas TLR-2 and TLR-6 heterodimers can respond to mycoplasma lipoproteins and peptidoglycan (Wetzler 2003).

Interestingly, the different dimers, TLR-2/-1 and TLR-2/-6 can even discriminate between tiny differences, such as the difference between triacyl-lipopeptide and diacyl-lipopeptide. What's more, various non-TLR molecules, such as adaptors, may influence the TLRs' specific repertoire (Akira and Takeda 2004). For example, LPS first needs to bind LPS-binding protein (LBP) in serum. This complex would serve to facilitate the binding of LPS to CD14 and TLR-4 on the cellular surface, which enhances both binding affinity and specificity.

To date, 10 TLRs have been identified in chickens (TLR1 (types 1 and 2), TLR2 (types 1 and 2), TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21) (Boyd *et al* 2001, Fukui *et al* 2001, Higgs *et al* 2006, Iqbal *et al* 2005, Kaiser 2007, Keestra *et al* 2007, Philbin *et al* 2005, Roach *et al* 2005 and Yilmaz *et al* 2005).

Chicken TLRs (chTLRs) were the first identified non-mammalian vertebrate TLRs. Based on the consensus sequences of *Drosophila* and mouse Toll families, Fukui and his colleagues first isolated chicken TLRs (type 1 and 2) with degenerate primers

from chicken bursa cDNA library (Fukui *et al* 2001). Because of their high homologies to human TLR-2, these two chicken TLRs were named TLR-2 type 1 and type 2. Further experiments showed that these TLRs were expressed in a wide range of tissues and organs especially in the connective tissues. The close location of these two genes on the same chromosome and their significant similarity suggested a duplication origin during evolution. However, it is interesting that only type 2 could recognize and signal both lipoproteins and LPS, but no tested microbial ligands were signaled by type 1 receptor. Chicken TLR-4 was identified two years later, and it was also expressed in almost all tissues tested (Leveque *et al* 2003). Other chicken TLRs (TLR-1 type 1 and 2, TLR-3, -5, -7, -15 and -21) were found in 2005 and 2006 respectively (Yilmaz *et al* 2005, Roach *et al* 2005 and Higgs *et al* 2006). In total, there are ten chicken TLRs identified so far including two different types (TLR-1 and -2 both have two types). Further investigation has revealed that the chromosomal locations of TLRs showed a high similarity between chicken and human. TLR-1, -2 and -3 are all located on chromosome 4 in both species except chTLR-1 is positioned on an un-determined micro- chromosome. In chicken, TLR-4, -5 and -7 are unlinked on different chromosomes 17, 3 and 1 respectively, which is very similar in human (chromosomes 9, 1 and X, respectively). The latest found chicken TLR-15 and -21 are located on chromosomes 3 and 11 respectively, but there was no homology in human or other mammals. Similar TLRs gene structures existed in both chicken and human, for example, most TLRs contain the same number of exons in both species, which shows a conservative evolution for the TLRs family (Yilmaz *et al* 2005). With improved bioinformatics tools and chicken genome database, more chTLRs may be identified and annotated. In mammals, CpG-ODN is a specific ligand of TLR-9.

However, chicken TLR-9 ortholog is not yet found. There may be other TLRs for CpG-ODN in chicken, but further experiments are needed to validate this hypothesis.

The chicken also shares similar evolutionary conservative signaling transduction pathway with mammals. A series of signaling pathway genes were identified by bioinformatic approaches. These genes included MyD88, Mal, IRAK-4, TRAF-6, TGF beta-activated kinase 1 (TAK1), TGF beta- activated kinase 1 binding protein 1 (TAB1), TAB2, inhibitor of NF-kB kinase alpha (IKK-alpha), IKK-beta, and Toll-interacting protein (Tollip).

Graaf *et al* (2005) stimulated human PBMCs and murine splenic lymphocytes with blastoconidia and hyphae of *Candida albicans*. They treated PBMCs with anti-TLR4 antibodies. They found that TLR4 mediates proinflammatory cytokine induction after *Candida* stimulation whereas *Candida* recognition by TLR2 leads mainly to anti-inflammatory cytokine release.

Binding between ligands and TLRs can induce signal transduction pathways and activate transcription factors within the host cells. The two common pathways are nuclear factor kappa-B (NFkB) pathway and the interferon regulatory factor (IRF) pathway. The first step in signal transduction requires the interaction between adaptor proteins and TLRs. The common adaptor proteins are MyD88 (myeloid differentiation primary-response protein 88), Mal (MyD88-adaptor-like protein, also known as TIR- domain-containing adaptor protein or TIRAP), TRIF (TIR-domain-containing adaptor protein inducing IFN-beta), TRAM (TRIF-related adaptor molecule) and SARM (sterile- alpha and HEAT-Armadillo motifs) (O'Neill *et al* 2003).

Different TLRs may recruit different adaptor proteins to induce different signaling

cascades. During the transduction pathways, a series of cytoplasmic intermediates, such as IRAK (IL-1 receptor-associated kinase) and TRAF (TNF receptor-associated factor), are also recruited and phosphorated, in turns. The transcription factors, such as NFkB and IRF-3, are activated in host cells (Akira and Takeda 2004). After being translocated into the nucleus, these activated transcription factors can bind to the transcription factor binding sites (TFBSs) on target genes and induce the expression of pro-inflammatory cytokines, chemokines and other immune-related factors, such as interleukin-6 (IL-6), IL-1, tumor necrosis factor-alpha (TNF-alpha) and interferon (IFN).

Most of the time, only a moderate level of inflammatory cytokines or chemokines are produced during the TLR signaling pathways, however, sometimes excessively inductive expressions of these immune-related molecules result in serious systemic disorders within the host, such as endotoxic shock or chronic rheumatoid arthritis.

Fortunately, multi-cellular organisms also develop mechanisms to regulate/modulate the TLR signal response and to maintain an inner homeostasis (Akira and Takeda 2004 and Dunne and O'Neill 2005). IRAK-M provides a good example to illustrate this negative feedback regulation. IRAK-M is one member of the IRAK family (Janssens and Beyaert 2003) and is only expressed in limited cell types such as macrophages and monocytes (Wesche *et al* 1999).

As compared to the wild type, the IRAK-M deficient mice exhibit an increased inflammatory response and cytokine production, and significantly reduced endotoxin tolerance with the challenge of TLR ligands. Close investigation has revealed that IRAK-M functioned to prevent the dissociation of IRAK-1 and IRAK-4 complexes from adaptor MyD88 and further inhibit the following phosphorylation cascades. IRAK-M increases

its expression with the activation of TLRs, which counteracts the inductive expression of pro-inflammatory cytokines via a negative feedback control (Kobayashi *et al* 2002). Suppressor of cytokine signaling (SOCS)-1 is another regulatory molecule and is induced to express by the pro-inflammatory cytokines. The activated SOCS-1 can also repress the TLR signaling cascades indirectly (Baetz *et al* 2004). SOCS-1 deficient mice are hypersensitive to LPS-induced shock, and increase the expression of inflammatory cytokines with LPS challenge (Kinjyo *et al* 2002). Recent studies also reveal other molecules functioning to down-regulation TLR signaling pathways such as single immunoglobulin IL-1-related (SIGGIR), MyD88 short (MyD88s) and TIR-containing proteins ST2 (Mansell *et al* 2006).

TLRs also play a critical role in adaptive immunity. It is well documented that the maturation of dendritic cells (DCs) involves the increasing expression of specific co-stimulatory molecules, which is dependent upon the activation of TLR signaling pathways (Banchereau and Steinman 1998 and Medzhitov 2001). During pathogen infection, the interaction of various ligands and TLRs on DCs induces the up-regulation of both co-stimulatory molecules and major histocompatibility complex (MHC) molecules, both of which facilitate the maturation of naïve T cells in adaptive immunity. As expected, the MyD88-deficient mice failed to produce IRF-gamma or active T helper type I cells with stimulation, because of the interruption of the TLRs signaling cascades (Schnare *et al* 2001). Also in this experiment, the immature DCs failed to be activated, which strongly illustrated the critical role of TLRs in the activation and induction of co-stimulation molecules in adaptive immunity. Also, the increased expression of various cytokines or chemokines induced by TLRs can significantly contribute to adaptive

immunity (Drakesmith *et al* 2000 and Pasare and Medzhitov 2004).

Takeuchi *et al* (2001) showed that macrophages from TLR6-deficient mice did not show any production of inflammatory cytokines in response to mycoplasma-derived diacyl lipopeptides. However, these cells showed normal production of inflammatory cytokines in response to triacyl lipopeptides derived from Gram-negative bacteria.

Dhinakar *et al* (2009) studied expression profile of toll-like receptor mRNA in an indigenous Aseel breed of chicken in india using reverse transcription polymerase chain reaction (RT-PCR). TLR 2 type 1 mRNA was expressed in lungs, liver, spleen, duodenum and caecal tonsils. TLR2 type 2 mRNA was expressed only in the lungs. TLR 3 mRNA was expressed in lungs, liver, spleen and caecal tonsils. TLR 4 mRNA was expressed only in lungs, liver and spleen. TLR 5 and TLR 7 mRNAs were expressed in all the tissues examined. With respect to tissues, heterophils and lungs expressed all the TLR mRNAs examined while kidneys expressed only TLR 5 and TLR 7 mRNAs.

2.3: Antimicrobial peptides (AMPs) and avian defensins

Antimicrobial peptides (AMPs) are very important innate immune effectors, and generally, they are either already stored in cell granules or easily induced to provide a prompt response during the early stage of pathogen's invasion. At physiologic conditions, the antimicrobial peptides exert multiple functions including a broad spectrum of antimicrobial activities (anti-bacteria, viruses, yeasts, protozoa and fungi), anti-steroidogenic activity, anti-cancer, chemotaxis and even inducing or regulating the adaptive immune system (Boman 2003, Zhang and Falla 2004, Ganz 2004 and 2005 and Brown and Hancock 2006). Nowadays, hundreds of different antimicrobial peptides have

been found in various species and these peptides are all small (less than 100 amino acids), cationic (rich in histidine, lysine, and arginine), amphipathic and evolutionally conserved (Hancock and Lehrer 1998). Also, the antimicrobial peptides all result from larger precursors with a signal leading sequence after transcription (Zasloff 2002). The exact antimicrobial mechanism of each peptide is variable and still not fully understood now, but the cationic characteristics are generally considered to have a close relationship with its function.

Antimicrobial peptides are a prevalent mechanism of host defense found throughout nature (Kaiser and Diamond 2000). These molecules are considered part of the innate immune system of all species (Kaiser and Diamond 2000, Ganz 2003, Lynn *et al* 2004 and Sugiarto and Yu 2004). Defensins are a family of antimicrobial peptides abundant in immune cells, white blood cells (specifically neutrophils), intestinal Paneth cells, and barrier epithelial cells, that engage in host defense (Ganz 2003).

There are many defensins that have been isolated from vertebrates and were classified into three subgroups, alpha- defensins, beta-defensins, and theta-defensins. Two of the subgroups, alpha-defensins and beta-defensins, have been identified in humans, cows and rodents. Theta-defensins so far have been identified only in leukocytes of rhesus monkeys (Zhao *et al* 2001 and Froy 2005). There is convincing evidence of conserved characteristics in all vertebrate defensins that indicates alpha-, beta-, and theta-defensins probably originated from a common ancestral defensin gene. Birds only have beta-defensins, and this leads to indicate they are probably the oldest of the three defensin subfamilies (Harwig *et al* 1994, Liu *et al* 1997, Zhao *et al* 2001 and Ganz 2003).

AMP binds preferentially to the negatively charged phospholipid bilayer of bacterial cells (Shai *et al* 2002). This is advantageous regarding decreasing the toxicity in any potential therapeutic environment. The lack of specific receptors make it difficult for bacteria to develop resistance to the peptide. Bacteria would have to alter the properties of their membrane as a whole rather than for specific receptors. Acidic polymers such as teichoic acids in Gram-positive (Neuhaus *et al* 2003) and phosphate groups present on lipopolysaccharides in Gram-negative bacteria (Muhle *et al* 2001) allow attachment of the peptide prior to formation of transmembrane pores and ultimately membrane permeabilization. Peptides that primarily possess antifungal activity tend to consist of neutral amino acids with regions of high polarity suggesting that a unique structure-activity relationship exists (Sung *et al* 2008).

Many facultative oral gram-negative bacteria are killed by human defensins (Miyasaki *et al* 1990). Histatins can be adsorbed into polyacrylic material to reduce Candida adhesion to the denture (Edgerton *et al* 1995). An additional effect of histatins is the inhibition of a proteinase from *Bacteroides gingivalis* (Nishikata *et al* 1991) and *in vivo* efficacy of histatins in dogs has been assessed experimentally (Paquette *et al* 1997). A promising development in AMPs-based gene therapy is the production of histatin 3 by infection with a histatin recombinant adenovirus, active on Candida strains (O'Connell *et al* 1996).

The physiological importance of antimicrobial milk peptides remains to be established, although it has been suggested that they may modulate the intestinal microflora when formed during milk digestion in vivo and protect host against invading microorganisms. According to the composition and structure, these antimicrobial peptides

can be divided into three main subfamilies: the cecropins, the cathelicidins and the defensins (Lehrer and Ganz 2002, Boman 2003 and Zanetti 2004). Cecropins are a family of linear alpha-helical peptides without cysteine residue and they were first found in insects in the early 1980s (Steiner *et al* 1981 and Boman, 2003).

Later, cecropins were also found in other organisms including mammals (Brogden *et al* 2005). These cecropins can lyse and kill foreign bacteria after integrating into the pathogen's membrane (Durell *et al* 1992). Cathelicidins were first identified in bovine myeloid cells in the 1990s and named because of their pro-region highly homologous to that of cathelin protein (Zanetti *et al* 1995). Also, cathelicidins were found in many other mammals including porcine, rabbit, mice, rats, and human (Tomasinsig and Zanetti 2005). The members of cathelicidins are conserved in their N- terminal domain but substantially heterogeneous in their C-terminal (Hancock and Diamond 2000).

Defensins are cysteine-rich antimicrobial peptides and typically contain six to eight cysteines motif, which can form three disulphide bonds (Schutte *et al* 2002 and Ganz 2003). Generally, defensins share a structure of triple-stranded beta-sheet with a beta- hairpin turn loop. Currently, various different defensins are found in a wide range of organisms including animals, insects and plants, and these defensins are widely distributed in host tissues/cells. The presence of defensins from lower to higher organisms significantly indicates their ancient origins, and that all these defensins may evolve according to a primordial immune mechanism (Raj and Dentino 2002). The first defensin was identified and purified from rabbit granulocytes (Selsted *et al* 1984). Later, from normal human neutrophils, three human defensins were found: human neutrophil peptide (HNP)-1, -2, and -3 and all of these peptides can effectively kill *Pseudomonas*

aeruginosa, *Staphylococcus aureus*, and *Escherichia coli* under experimental conditions (Ganz *et al* 1985).

The insect defensins were first purified from the cell culture medium of a flesh fly, *Sarcophaga peregrine* (Matsuyama and Natori 1988), and currently defensins are found in almost all insect species investigated. Mendez *et al* (1990) found a new basic and sulfur-rich polypeptide from barley endosperm and named it gamma-hordothionin.

Interestingly, these defensins show considerable variations in their sequence and structure perhaps because of selective pressure within different species or different living conditions during long time evolution (Oppenheim *et al* 2003). Based on species specificity, disulfide bonds connectivity and cysteine spacing, these defensins can be grouped into five families: alpha, beta, theta, invertebrate and plant defensins (Raj and Dentino 2002). The first three mainly exist in vertebrates and are currently under intensive study.

Alpha-defensins only exist in mammals and they were first identified from human neutrophil granules (Ganz *et al* 1985). Generally, alpha-defensins contain 29-35 amino acids and their expression patterns are both species- and tissue-specific. An inbred laboratory mouse could express nearly 20 alpha-defensins in paneth cells, whereas it didn't express any in polymorphonuclear (PMN) leukocytes (Eisenhauer and Lehrer 1992). However, rats are more similar to human and express several alpha-defensins in both paneth cells and PMN leukocytes (Eisenhauer *et al* 1989 and 1990). Currently, there are totally six alpha-defensins isolated from human and all these defensins are closely located on chromosome 8 (Linzmeier *et al* 1999). HNP-1 to -4 are produced in neutrophils, with HNP-4 expressed lower as compared to other three. Human alpha-

defensin-5 and -6 (HD-5 and -6) are identified as enteric defensins in paneth cells of the small intestine (Jones and Bevins 1992). The expressions of these alpha-defensins are regarded as constitutive in human (Cowland and Borregaard 1999).

Theta-defensins are only found in limited species such as rhesus monkey (*Macaca mulatta*), and the first theta-defensin was identified in 1999 from the granules of neutrophils and monocytes (Tang *et al* 1999). Linked with two alpha-defensin-like sequences, the theta-defensin shows a distinctively cyclic structure of 18 amino acid residues, which confers it higher anti-viral activity than alpha-defensins (Lehrer 2004). However, human only contain a mutated pseudogene of theta-defensin and this gene silences its expression (Levy 2004). The beta-defensins are the largest group of defensins and exist in a wide range of vertebrates including mammals, reptiles, fish and birds (Sugiarto and Yu 2004 and Lehrer 2004). In 1991, the beta-defensin was first identified from bovine tracheal epithelia, but was named as tracheal antimicrobial peptide (TAP) at that time (Diamond *et al* 1991).

With more similar peptides found, this group of defensins was discovered to differ from other peptides in both consensus sequences and tri-disulfide motifs. So, they were renamed beta-defensins (Selsted *et al* 1993). These beta-defensins contain a similar 3-dimensional structure (three intra-molecular disulfide bonds forming a beta-sheet) to that of alpha-defensins. But beta-defensins are generally larger and the spacing and connection of cysteine residues are different from those of alpha group.

The structure of beta-defensins is characteristic with a short alpha-helix (or turn) juxtaposed with two or three anti-parallel beta-strands. However, there are still limited variations in the secondary structure of beta-defensins suitable for specific functions. For

example, bovine beta-defensin-12 contains a turn-like configuration and is absent the short helix (Torres and Kuchel 2004). From phylogenetic analysis, the beta-defensins are regarded as arising much earlier than alpha- and theta-defensins. Now it has been conceived that both alpha- and beta-defensin genes originated from a beta-defensin-like gene by a series of duplications (or mutations) and selection pressure, and theta-defensins arose from a pre-existed alpha-defensin gene (Nguyen *et al* 2003, Xiao *et al* 2004 and Radhakrishnan *et al* 2005).

The expressions of beta-defensins are generally induced by foreign stimulations or signals at transcriptional level in various tissues. IL-1 and TNF-alpha are important pro-inflammatory cytokines regulating defensins' expression (Harder *et al* 2000 and Abbas and Lichtman 2003). In human, the expressions of most HBDs (human beta-defensins- 2, -3 and -4) are highly inducible (Harder *et al* 2001, Garcia *et al* 2001 and Schutte and McCray 2002). In fact, HBD-2 has recently been intensively investigated and much information about its regulation mechanism has already been elucidated. HBD-2 was first identified from the surface of lung epithelia in 1998 (Bals *et al* 1998), but it was only detected in lung disease patients instead of the normal ones (Singh *et al* 1998). Numerous experiments had already proved that HBD-2 was induced to express by various pro-inflammatory signals including cytokines and bacterial chemicals.

The signaling transduction pathways involving the inductive expression of HBD-2 are very complex, and it is generally regarded nuclear factor (NF)kB playing the most important role in this regulation. Blocking the activation of NF-kB completely inhibits the inducible expression of HBD-2 by IL-1-alpha in intestinal epithelia (O'Neil *et al* 1999). Sequence analysis revealed that several putative transcription factor binding sites

(TFBSs) for NFkB were located at both 5' proximal promoter region and introns of HBD-2 gene. Mutation or deletion of -208nt of HBD-2 gene (the NFkB binding site) would decrease or even silence its transcription (Wada *et al* 2001). Also, from the experiments of luciferase reporter gene, the activator protein (AP)-1 binding site (positions -127 to -121) was found in the promoter region, which meant that AP-1 also played a critical role in inducing HBD-2's expression with the stimulation of IL-1-beta or PA (pseudomonas aeruginosa) (Wehkamp *et al* 2006).

Beta-defensins exert multiple microbicidal functions in innate immune response to a wide range of pathogens and infections, and the deficiency of beta-defensins is associated with various dysfunctions or diseases such as inflammatory bowel diseases in human (Cobrin and Abreu 2005). However, the killing abilities depend upon the salt concentration as well as other physiological conditions. The high salt environment would inhibit the microbicidal functions of beta-defensins (Bals *et al* 1998). The positive-charged beta -defensins would effectively interact with the negative- charged microbial membrane components. This electrostatic interaction permeabilizes the microbial cytoplasmic membrane and kills the invasion microbes in a multimer manner (Raj and Dentino 2002 and Chen *et al* 2006). Besides direct killing mechanisms, beta-defensins also exert a serious of other functions such as chemotaxis and immune regulation. For example, HBD-1 and -2 are selectively chemotactic for memory T cells and immature dendritic cells (Yang *et al* 1999).

Currently, antimicrobial peptides have become very popular in medical field because of their unique microbicidal mechanisms differing from those of classic antibiotics. Extensive clinical use of conventional antibiotics results in potential dangers

of drug resistance or side effects. However, beta-defensins are produced by vertebrates themselves.

Lynn *et al* (2007) proposed to adopt the numbering system used by Xiao *et al*. (2004) as this system is currently used by the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/). To be consistent with the usual nomenclature of other vertebrate beta- defensins, however, we also propose that the term gallinacin be dropped and instead of that, the term "avian beta-defensin" (abbreviated to AvBD) be used to describe these molecules.

These natural antimicrobial peptides do not involve new selection pressure and will surely be beneficial to the host. Since the first chicken beta-defensin was found in 1994 from leukocytes (Harwig *et al* 1994). To date, 14 avian beta-defensins have been isolated either from heterophilic granulocytes or discovered by in silico analysis and were found to be constitutively or inducibly expressed following infection with bacteria or their components, has also been shown to occur in chickens (Lynn *et al* 2004 and 2007 and Xiao *et al* 2004).

All of the defensin genes are densely located in an 86-Kb nucleotide fragment on chromosome 3q3.5-q3.7, which contrasts with several clusters of beta-defensin genes on different chromosomes in other vertebrates such as human and mouse (Schutte *et al* 2002). Generally, beta-defensins are more important in innate immune response for avian than mammal because of the lack of oxidative mechanisms in avian heterophils (Sugiarto and Yu 2004).

Antimicrobial peptides are leading in the development of novel biocidal agents at the moment when classical antibiotics are under intense pressure from emerging

resistance (Laverty *et al* 2011). In addition to their antimicrobial role, AMPs also serve as important effector molecules in inflammation, immune activation, and wound healing (Huang *et al* 2004). Nearly 1200 antimicrobial peptides have now been identified (Matejuk *et al* 2010).

Nature has strategically placed antimicrobial peptides as first line of defenses between the host organism and its surrounding environment, as these peptides inhibits over a wide range of infectious microbes deleting the effect of toxicity to the host organism.

Domesticated animals have a large variety of antimicrobial peptide that serve as natural innate barriers limiting the microbial infection and also acts as an integral component in response to inflammation. The multiple modes of action utilized by AMPs reduce the ability of microorganisms to develop resistance, with minute activity shown against bacteria resistant to standard antibiotics (Giuliani *et al* 2007).

With the focus on expanding and/or refining resources against infection in an era of antibiotic resistance, in the recent years vast area of work has been invested in localizing new antibiotic peptide sequences and improving their potency and selectivity. Few reasons which can be counted to spice up the antibiotic resistance in microrganisms includes the use of low levels of antibiotics as growth promotors in animal feeds (Witte *et al* 2000) and the extensive use of antibiotics to treat human or animal infections (Diekema *et al* 2000) . Thus, at present, advances in production and development of both synthetic and recombinant cost effective antibiotic peptides are on its way of progress.

Broad spectrum AMP's target and lyse the membrane of the microbes, yet these peptides frequently have less proclivity to lyse mammalian cell membranes such as those

of red blood cells despite targeting and lysing microbial membranes, the potencies and spectra of activities of the AMPs against different classes of microbes vary and depend on the membrane composition of the pathogen and the structure of the peptide. The antifungal activity of AMPs is multifactorial. For example, AMPs stimulate the immune system in mammals by several mechanisms: 1) activation of T-cells, 2) stimulation of Toll-like receptors, 3) amplifying phagocyte action, 4) activation of dendritic cells, and 5) chemo-attraction of neutrophils (Biragyn *et al* 2001 and Yang *et al* 2002).

Moreover, these activated cells and receptors may reduce the growth of fungi in vivo by modifying levels of various cytokines, chemokines, and integrins (Durr *et al* 2002 and Hancock *et al* 2002). It helps in providing immune-surveillance against pathogens and maintaining a healthy floral milieu. Studies have shown the potential of antimicrobial cationic peptides in cancer and gene therapy (Leng *et al* 2005). As a result, the peptides offer promise for future treatment of infectious diseases in a diverse range of organisms

In addition to cysteine-rich AMPs, there are other prominent subgroups of AMPs in animals characterized on the basis of primary or secondary peptide structure (Brogden 2005). One subgroup is characterized by an abundant number of cationic peptides containing basic and/or hydrophobic residues at a high ratio and many of them conform into a-helical structures in membrane mimetic environments. Examples of these subgroups include mammalian cathelicidins, amphibian magainins and maximins, and insect cecropins. Some members of this subgroup, such as cathelicidins, are rich in certain residues such as proline (e.g. porcine PR-39), phenylalanine (e.g. porcine prophenins) and tryptophan (e.g. bovine indolicidin), which may contribute to their

'multi-hitting' model of antimicrobial responses (McPhee *et al* 2005, Hancock and Sahl 2006 and Hale and Hancock 2007). The second subgroup of AMPs includes antimicrobial fragments derived from large proteins such as lactoferricin from lactoferrin and the antimicrobial domain of lysozyme (Brogden 2005). Potent activity against a broad spectrum of micro-organisms including bacteria, fungi, enveloped viruses and tumor cells has been observed in members of these animal AMPs along with multiple roles in respect to immunoregulation and cell signaling (Zaiou 2007).

The overall antimicrobial effect of an AMP in vivo, which is manifested by suppression/elimination of infection by a pathogen, can result from both its direct antimicrobial activity and indirect immune regulatory functions. In this context, most AMPs in higher vertebrates, such as mammalian defensins and cathelicidins, have been shown to be multifunctional and because of this property are often referred to as host defense peptides (Zaiou 2007). Immunoregulatory functions exerted by mammalian antimicrobial host defense peptides includes chemoattractant activity for immune cells, inhibition of oxidative burst of phagocytes, promotion of angiogenesis and wound healing, regulation of development and function of male reproductive cells, and induction of autoimmunity. Although these multifunctional properties may increase the drug development potential of AMPs, some may also cause limitations in the development of antibiotics. Other challenges to AMP-based drug development include cytotoxicity and the higher cost of peptide synthesis (Scott *et al* 2007).

Although microbial resistance is considered less likely for AMPs than conventional antibiotics, some mechanisms of resistance to AMPs have been identified (Gunn 2008 and Kraus and Peschel 2008), this should be considered in developing and

using AMP-based drugs. Peptide therapeutics represents a novel class of therapeutic agents. Beta- defensins and their mimics form a diverse class of antibacterial agents currently validated in preclinical and clinical settings for the treatment of infections caused by antimicrobial-resistant bacteria. However, due the lack of extensive study related to naturally occurring anti-microbial peptides including defensins, isolation of such peptides often suffer from a variety of pharmacokinetic shortcomings and poor bioavailability.

2.4: Regulatory and signaling pathway of innate immunity

Understanding the complex mechanisms of regulating gene expression is one of the greatest challenges faced by modern molecular biology. Two important functional elements in a genome are transcription factor genes (TFs) and genomic sequences, transcription factor binding sites (TFBS) to which TFs bind (Bulyk 2003). Gene expression is regulated by binding of transcription factors to the promoter. Over the past few years, a number of beta-defensins have been identified in various animals and found to exhibit constitutive and inducible gene expression. For example, inducible expression of beta-defensins can be detected in blood after stimulation with LPS. NFκB proteins are a family of inducible transcription factors that allow cells to respond to extracellular stimuli, e.g. LPS.

The stimulation of foreign pathogens induces and increases the transcription of pro-inflammatory cytokines, such as IL-6, IL-1 and TNF- alpha, mainly in local cells (macrophages or endothelia). Generally, the IL-1-like cytokines will activate and translocate the transcription factors NFkB and activating protein-1 (AP-1), whereas the

IL-6-like cytokines function through JAK/STAT (Janus kinases/signal transducers and activators of transcription) pathway. Also, a common pathway shared by both IL-1- and IL-6-like cytokines is through mitogen activated protein kinases (MAPKs) and transcription factor NF-IL-6 (Wu *et al* 2003). These regulatory molecules include glucocorticoids and other anti- inflammatory cytokines such as IL-4 and IL-10 (Ceciliani, *et al* 2002).

Katial *et al* (1998) studied the production of IL-12, IFN-γ, TNF-α, TNF-β, IL-5 and IL-10 *in vitro* by stimulation of PBMCs, from immunocompetent subjects, with mitogens, concanavalin A, phytohemagglutinin, pokeweed mitogen and *Staphylococcus aureus*.

The first beta-defensin described was isolated from the tracheal epithelium of cattle, in which its expression is inducible by LPS through a CD14-dependent signaling pathway and the transcription of the bovine tracheal antimicrobial peptide (TAP) gene was found to be regulated by transcription factors such as NF-κB (Tsutsumi and Nagaoka 2002).

TLRs are type I orphan receptors with an extra cellular portion containing LRR, and a cytoplasmic domain significantly similar to the intracellular portion of the IL-1R. These observations suggest that IL-1R and TLR may use an analogous molecular framework for signaling. The IL-1R and TLR family signal via shared downstream signaling molecules. They include the adaptor molecule MyD88, IL-1R-associated protein kinases (IRAKs), the transforming growth factor (TGF)-β-activated kinase (TAK1), TAK1-binding protein1 (TAB1) and 2 (TAB2), and the tumor necrosis factor receptor associated factor 6 (TRAF6) (O' Neill 2002).

Triggering of the IL-1R or TLR causes the adaptor protein MyD88 to be recruited to the receptor complex, which in turn promotes association with the IL-1R-associated kinases, IRAK-4 and IRAK-1. During the formation of this complex, IRAK4 is activated, leading to the hyper phosphorylation of IRAK-1, which then induces the interaction of TRAF6 with the complex. The association of IRAK-4.IRAK-1.TRAF6 causes some conformational change in one or more of these factors, leading to their disengagement from the receptor complex. The IRAK-4.IRAK-1.TRAF6 complex then interacts at the membrane with another preformed complex consisting of TAK1, TAB1, and TAB2. This interaction induces phosphorylation of TAB2 and TAK1, which then translocate together with TRAF6 and TAB1 to the cytosol. TAK1 is subsequently activated in the cytoplasm, leading to the activation of IKK. Inactive IKK sequesters NF-κB in the cytoplasm, but activation leads to phosphorylation and degradation of IκB and consequent release of NF-κB, a ubiquitous transcription factor that regulates the transcription of various genes during inflammatory and immune responses. Activation of TAK1 also results in the activation of MAP kinases and c-Jun NH2-terminal kinase (JNK).

Wang *et al* (2001) studied the mechanism of micrococci and peptidoglycan (PGN) induced transcription of IL-8 in HEK293 cells expressing TLR2 and CD14 but not TLR1 or 4. They concluded that TLR2- mediated signal transduction pathways involves IRAK, MyD88, TRAF6, IKKα, IKKβ, NIK and NF-kB and MyD88 is an essential component in this pathway.

Although MyD88 plays a critical role in TLR signaling, there is a difference in the signaling pathways triggered by LPS and by other bacterial components. Activation of NF-κB and MAP kinases by mycoplasmal lipopeptide is completely abolished in

TLR2- or MyD88-deficient macrophages. However, LPS activation of MAP kinases and NF-κB remains intact in MyD88-deficient macrophages, although it is delayed compared with that in wild-type mice. This indicates that the TLR4-mediated response to LPS may involve both MyD88-dependent and -independent pathways, each of which leads to the activation of MAP kinases and NF-κB.

Kawai *et al* (2001) performed subtractive hybridization studies to show that interferon-inducible genes including IP-10, a CXC chemokine, are induced in MyD88-deficient macrophages in response to LPS.

2.5: Heterophils in birds

The avian heterophil has been declared a counterpart to the neutrophil in mammals. Heterophils also secrete a wide variety of enzymes, chemokines, and cytokines, which help to amplify immune responses by recruiting other cells to the site of inflammation, thereby contributing to an early resistance to infection (Kogut *et al* 1998, Nikolaus *et al* 1998, Rath *et al* 1998, Oliveira *et al* 1999 and Brandt *et al* 2000).

Although there are many similarities between these two granulocytes, there also are important differences. The heterophilic inflammatory response in avian species more closely resembles the reptilian response than the mammalian response (Montali 1988). Heterophils are the predominate granulated leukocyte in the acute inflammatory response in gallinaceous birds. Heterophils are highly phagocytic and are capable of a broad spectrum of antimicrobial activity. They accumulate in inflamed tissue, causing tissue damage and forming heterophil granulomas that are morphologically similar to inflammatory lesions in reptiles. The avian heterophil lacks myeloperoxidase and depends primarily on nonoxidative mechanisms for antimicrobial activity. The

hematologic response of turkeys to intravenous administration of LPS has recently been characterized. The influx of heterophils is the first line of cellular defense in the avian respiratory tract because there is no resident population of pulmonary macrophages (Toth and Siegel 1986 and Ficken and Barnes 1989). When compared to the experimental work on neutrophils and the inflammatory response in mammals, similar work in birds and reptiles is rather meager. Therefore, much about avian and reptilian heterophils has been inferred from neutrophil studies in mammals.

The beta-defensins found in heterophil granules can kill a wide variety of bacterial pathogens and are a major component of the heterophil antimicrobial arsenal. Heterophils form the first line of cellular defense against invading microbial pathogens in the lungs and air sacs where resident macrophages are lacking.

Because preventive medicine is emphasized in poultry medicine and therapies to modify or ameliorate inflammation are not very practical in poultry species, studies to understand the heterophil and the acute inflammatory response in avian species have lagged behind those in humans and other mammals. More recently, it has become apparent that the heterophil and other first-line defense mechanisms activated during the acute inflammatory response have an important role in innate disease resistance. It is important to understand these early defense mechanisms not only to avoid compromising their effectiveness by management practices but also to find ways in the future to manipulate these mechanisms to better protect birds from infectious diseases. Investigations comparing the susceptibility of pathogenic and nonpathogenic bacteria to bactericidal activity of avian defensins are needed to determine whether resistance to these antimicrobial peptides is associated with virulence for avian pathogens. Resistance

of S. typhimurium to killing by defensins does appear to be associated with virulence in a mouse model (Groisman *et al* 1992 and Parra *et al* 1993). The avian beta-defensins are probably just one group of many heterophil antimicrobial mechanisms that await further study. In the process of purifying these beta-defensins, other antimicrobial fractions from heterophil granules have been detected, but have not yet been purified or characterized.

2.6: Lipopolysaccharide (LPS) stimulation of cells

As early as 1996, bovine beta-defensins (TAP) were found to significantly induced with the challenge of LPS in cultured tracheal epithelial cells (Diamond *et al* 1996 and Russell *et al* 1996). In human, HBD-2 showed the same characteristics and was induced to express with the treatment of LPS or pro-inflammatory cytokines such as IL-1-beta or TNF-alpha (Singh *et al* 1998, Mathews *et al* 1999 and Hao *et al* 2001). HBD-3 and -4 are also under similar regulation mechanisms. HBD-3 increases its expression with the stimulation of bacteria, TNF-alpha and IL-1-beta, and HBD-4 is induced when responding to PMA (phorbol myristate acetate), LPS and TNF-alpha (Garcia *et al* 2001, Yanagi *et al* 2005 and Vankeerberghen *et al* 2005).

Lin *et al* (2000) cloned and functionally characterized mouse TLR2 from 3T3-L1 adipocytes. They found that TLR2 synthesis is strongly induced in the adipocytes by LPS, TNF-α and the yeast cell wall extract zymosan. Further, TLR2 undergoes a lengthy intracellular maturation process with a half-life of exit from the ER of approximately 3 h.

Wang *et al* (2000) studied the potential interaction between LPS and PGN in the induction of the sepsis- associated cytokines, TNF- α, IL-6 and IL-10 in whole human blood by measuring their plasma levels by enzyme immunoassays and a TNF bioassay.

They found that co-administration of PGN (10 µg/ml) or MDP (1ug/ml) with LPS(10ng/ml) caused significantly elevated values of TNF- α and IL-6 in blood that could not be obtained by the sum of values obtained by each stimulant alone or by 3- fold higher doses of either bacterial components alone. They suggested that PGN and MDP prime human whole blood leucocytes for LPS-induced release of pro-inflammatory cytokines.

Faure *et al* (2000) showed that LPS, TNF-α and IFN-γ induce TLR2 expression in both human dermal micro vessel EC and HUVEC. LPS and IFN-γ act synergistically to induce TLR2 expression in EC and LPS- induced TLR2 expression is NF-kB dependent.

Pulendran *et al* (2001) demonstrated that interaction of *E. coli* LPS with TLR4 induces production of IL-12p70 while the recognition of *P. gingivalis* LPS by TLR2 is unable to induce IL-12p70 release and favors a Th2-type response.

Dillon *et al* (2004) investigated into TLR-2 knockout mice (TLR-$2^{-/-}$) and myeloid differentiation factor 88 (MyD88) knockout (MyD$88^{-/-}$) mice to demonstrate that different Toll-like receptor (TLR) ligands induce distinct dendritic cell (DC) activation and immune responses in vivo. Thus, *Escherichia coli* LPS (TLR-4 stimulus) activates DCs to produce abundant IL-12(p70), but little IL-10, and stimulates Th1 and T cytotoxic 1 (Tc1) responses. In contrast, Pam-3-cys (TLR-2 stimulus) elicits less IL-12(p70), but abundant IL-10 and favors Th2 and Tc2 responses**.**

Ajuwon *et al* (2009) investigated the response to PGN from *S. aureus* in differentiated 3T3-LI adipocytes and used real- time PCR to quantify the expression of IL-6, adiponectin receptors, TLR2 and TLR4. They concluded that both PGN and LPS robustly induce TLR2 mRNA expression whereas TLR4 mRNA is weakly induced by

LPS only. They further found that PGN downregulates the expression of adiponectin receptors.

2.7: Quantitative gene expression studies related to innate immunity in chicken

The ability to sequence entire genomes has stimulated research directed not only at producing DNA sequence, but also at defining the function of genes on a genome-wide level. Given that genes with related functions are likely to be regulated together, techniques that evaluate global gene expression provide a mechanism for the initial identification and clustering of novel gene sequences with related functions. In the last two decades techniques for the evaluation of gene expression have progressed from methods developed for the analysis of single, specific genes to techniques focused on identifying all genes that differ in expression between or among experimental samples (Moody 2001).

Leveque *et al* (2003) proposed that TLR4 is linked to resistance to infection with *Salmonella enterica* serovar Typhimurium in chickens on northern blot analysis of *TLR4* expressed in tissues including brain, thymus, kidney, intestine, muscle, liver, lung, bursa of fabricius, heart, and spleen.

Hirschfeld *et al* (2001) were the first to suggest that differential cytokine patterns are released when various TLRs are engaged by lipopolysaccharides (LPS) from different species e.g stimulation with *Escherichia coli* LPS, a ligand for TLR4, led to release of large amounts of tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-12p40, and IP-10 (gamma-interferon-inducible protein 10), whereas *Porphyromonas gingivalis* LPS, a TLR2 ligand, induced moderate amounts of TNF and IL-1β and no production of IL-12p40 or IP-10 .

Expression and function of Toll-like receptors in chicken heterophils was demonstrated by Kogut *et al* (2005) using TLR agonists, including flagellin (from Salmonella typhimurium, FGN), peptidoglycan (from Staphylococcus aureus, PGN), ultra-pure lipopolysaccharide (from Salmonella minnesota, LPS), the synthetic double stranded RNA analog [poly(I:C)], and the guanosine analog, loxoribine (LOX) to directly induced both an oxidative burst and a degranulation response. all induced an up-regulation of expression of mRNA of the pro-inflammatory cytokines IL-1b, IL-6, and IL-8, whereas both poly(I:C) and LOX induced a down-regulation of these cytokine mRNAs. The broad TLR expression profile in heterophils reflects their principal role as first line effector cells in avian host defense against bacterial, viral, fungal, and parasitic infections.

Iqbal *et al* (2005) determined the mRNA expression patterns for seven chicken TLRs (chTLR) in a wide range of chicken tissues, isolated immune cell types and cultured cells. Some of the chTLR were expressed in most tissues (chTLR1/6/10, chTLR3, chTLR4 and chTLR5), whereas others exhibited more restricted expression patterns (chTLR2 type 1, type 2 and chTLR7). Similarly distinct patterns of chTLR expression were seen with innate and adaptive immune cell types isolated from peripheral blood or spleen and with cultured cells of somatic or immunological origin. An understanding of the TLR repertoire for different tissues, immune cell subsets and cultured cell types allows more refined interpretation of immune induction in response to chicken pathogens.

Kaiser *et al* (2006) studied cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to *Salmonella enterica* serovar. Interleukin-2,

interleukin-6 (*IL-6*), *CXCLi2*, an transforming growth factor-*β*4 (*TGF-B4*) messenger ribonucleic acid expression was measured by quantitative reverse transcription-PCR assays in PBMC from 3 chicken lines (broiler, Leghorn, Fayoumi) after in vitro exposure to *S*. Enteritidis from cultures were harvested 2 or 4 h of exposure and found that exposure to *S*. Enteritidis down-regulated *IL-6*, *CXCLi2*, and *TGF-β4* but not interleukin-2 mRNA expression. No significant genetic line or exposure time effects were detected. These findings demonstrate that exposure of chicken PBMC to *S*. Enteritidis can induce a rapid change in both proinflammatory (*IL-6*, *CXCLi2*) and anti-inflammatory (*TGF-β4*) cytokine gene expression.

Abasht *et al* (2009) investigated the acute effect of *Salmonella* Enteritidis challenge on TLR mRNA expression in cecum and spleen of birds from 3distinct genetic lines. Chicks from broiler, Leghorn, and Fayoumi lines were inoculated or mock-inoculated with *Salmonella* Enteritidis. The mRNA expression levels of TLR2, TLR4, and TLR5 genes were assessed by quantitative reverse transcription-PCR of cecum and spleen tissue harvested at 2 or 18 h post inoculation. There were no significant genetic line effects on TLR mRNA expression in spleen or cecum of mock-infected birds, or in the cecum of infected birds. Genetic line effect was significant ($P < 0.05$) on TLR mRNA expression in the spleen of *Salmonella* Enteritidis-infected birds. The Fayoumi line had higher TLR2 and TLR4 expression than Leghorn, higher TLR2 mRNA expression than broiler, and the broiler line had higher TLR5 expression than Leghorn and Fayoumi. In *Salmonella* Enteritidis-infected birds, the TLR2 expression in both cecum and spleen and TLR4 expression in spleen were significantly higher at 18 h PI than 2 h PI. The results demonstrate a significant genetic line effect on TLR expression in the spleen of

Salmonella Enteritidis-infected birds, which may partly explain genetic variability in immune response to *Salmonella* Enteritidis

Musa *et al* (2009) studied the expression profile of TLRs and cytokines to determine the role of LPS in the peripheral blood lymphocytes of chicken. Semi-quantitative RT-PCR studied suggested that lymphocytes express mRNA of TLRs (TLR1/6/10, TLR3, TLR4, TLR5 and TLR7) and interleukins (IL-1, IL-8, IL-18 and TGF-4). LPS significantly ($P<0.05$) induced the expression of TLR4, IL-1, IL-8 and IL-18, and non-significantly induce the mRNA expression of TLR1/6/10, TLR5 and TGF-4, thus proving that LPS plays an important role in the innate immune response of lymphocytes to pathogen.

Dhinakar *et al* (2009) studied expression profile of toll-like receptor mRNA in an indigenous Aseel breed of chicken in India using reverse transcription polymerase chain reaction (RT-PCR). TLR 2 type 1 mRNA was expressed in lungs, liver, spleen, duodenum and caecal tonsils. TLR2 type 2 mRNA was expressed only in the lungs. TLR 3 mRNA was expressed in lungs, liver, spleen and caecal tonsils. TLR 4 mRNA was expressed only in lungs, liver and spleen. TLR 5 and TLR 7 mRNAs were expressed in all the tissues examined. With respect to tissues, heterophils and lungs expressed all the TLR mRNAs examined while kidneys expressed only TLR 5 and TLR 7 mRNAs.

Ramasamy *et al* (2010) quantified the expression of TLR3, TLR4, TLR5 and TLR7 in Aseel, Kadaknath, Naked neck, Dwarf and White Leghorn lines by quantitative real-time PCR. White Leghorns expressed significantly ($P<0.01$) higher levels of TLR3 mRNA compared to other lines. TLR4 and TLR5 mRNA were significantly highly expressed in Kadaknath line. Among the TLRs investigated TLR5 was more expressed in

all lines studied. TLR7 was highly expressed in indigenous chicken Aseel and Kadaknath than other lines. Dwarf chicken expressed significantly ($P<0.01$) lower levels of all TLRs investigated. On the basis of the study they concluded that the differential expression of TLR mRNA in the heterophils of indigenous and other chicken breeds might contribute to their variable disease resistance/susceptibility.

Derache *et al* (2009) purified three biologically active beta-defensins by chromatography from chicken bone marrow: avian beta-defensins AvBD-1, AvBD-2 and AvBD-7. Their antibacterial activities were assessed against a large panel of Gram-positive and Gram-negative bacteria. All three defensins displayed similar activity against Gram-positive strains, but AvBD-1 & AvBD-7 exhibited stronger activity against Gram- negative bacteria.

Ebers *et al* (2009) determined mRNA expression profiles of 14 avian beta-defensins (AvBDs) in primary chicken oviduct epithelial cells before and after infection with *Salmonella enterica*. The infection temporarily inhibited expression of certain AvBDs but induced expression of other minimally expressed defensins.

Ramasamy *et al* (2012) studied differential gene expression of antimicrobial peptides beta defensins (AvBDs 1–14) in the gastrointestinal tract of Salmonella serovar Pullorum infected broiler chickens. Quantitative real-time PCR analysis revealed significant ($P<0.05$) upregulation of AvBD3, 4, 5, 6 and 12 and a significant ($P< 0.05$) down regulation in the expressions of AvBD10, 11, 13 and 14 in one or few GI tissues, while no significant changes were observed for AvBD1, 2, 7, 8 and 9 gene expressions in any of the GI tissues investigated upon infection with S. Pullorum. Most substantial

change in gene expression was found for AvBD5, being significantly ($P<0.01$) upregulated in most of the GI tissues investigated.

CHAPTER III

Materials and Methods

The present study has been accomplished in three parts: culturing of heterophils, RNA isolation and differential expression study of innate immunity related genes. Avian beta-defensins (AvBDs), Toll-like receptors (TLRs), transcription factors and pro-inflammatory cytokine were analyzed in broiler, layer and Aseel stocks of chickens, using quantitative real-time PCR, SYBR Green chemistry.

3.1: Birds and collection of blood

The study was conducted on birds maintained at Poultry Breeding Farm, GADVASU Ludhiana. The broiler strain PB1 had undergone mass selection primarily for juvenile body weight over more than 35 generations. Concomitantly the strain had been improved for maternal attributes (e.g. egg production and reproduction). The layer chicken strain, PL2, had been selected for over 36 generations, primarily for egg production to 40 weeks of age. Other traits for which the strain had undergone mild selection included egg, mortality, fertility and hatchability. The stock of Aseel had been maintained without any intentional selection.

Six, apparently healthy, adult birds of three distinct genetic groups viz, strain of broiler, strain of layer and a stock of indigenous breed Aseel were randomly chosen for peripheral blood collection from the wing vein. The birds were from same age group, and had been reared under standard managemental regimen.

About 1-2 ml of peripheral blood was aseptically collected from the wing vein of each bird using 24G needle and transferred to 15 ml sterile tubes containing EDTA (EDTA, 1.5 mg/ml of blood, pH-8) for heterophil isolation and further culture.

3.2: Isolation of heterophils

Avian heterophils were isolated from the peripheral blood of chickens as described previously (Kogut *et al* 2005) using Ficoll-Hypaque (Sigma-Aldrich, USA) gradient 1.077/1.119 interfaces as per the following protocol:

1. The collected blood was diluted in equal volume of phosphate buffered saline (PBS, pH 7.4).
2. In a conical, pre-sterilized, 15 ml centrifuge tube 1.5 ml of Histopaque 1119 (Sigma-Aldrich, USA) was poured. Carefully layer 1.5 ml of Histopaque 1077 (Sigma-Aldrich, USA) onto the Histopaque 1119.
3. 3 ml of the diluted blood was over layered onto it slowly from the side of the tube so as to avoid mixing, to create a sharp interphase.
4. The tube was centrifuged at 250 x g for 60 minutes, break off, at room temperature in a swinging bucket rotor centrifuge.
5. After centrifugation the 1077/1119 interphase and 1119 band contain the heterophils, which were aspirated using a clean and pre-sterilized micro tip and transferred to a clean centrifuge tube
6. Equal volume of RPMI 1640 (Sigma-Aldrich, USA) was added to the separated heterophils and mixed gently.
7. The tube was centrifuged at 200 x g for 10 min, at room temperature. This washing removes Histopaque. The washing with RPMI 1640 was repeated twice.

3.3: Recovery and viability of heterophils

The recovery and viability of the isolated heterophils was determined by haemocytometer counting using the trypan blue dye exclusion method (Kogut *et al* 2005), which was performed as:

i) 10 µl of cell suspension was mixed with 10 µl of 0.4 % trypan blue and incubate for 3-5 min at room temperature.

ii) 10 µl of the trypan blue cell mixture was injected beneath the cover slip on a haemocytometer.

iii) The haemocytometer was placed on the stage of a binocular microscope and the cells focused.

iv) The unstained (viable) and stained (dead) cells were counted from the central large squares of haemocytometer and at least 100 cells were counted.

v) The number of total viable cells was calculated as:

Total viable cells = Viable cells per square x 2 x10, 000 x total volume of cell suspension.

$$= 40 \times 2 \times 10,000 \times 3$$

$$= 2.4 \times 10^6 / \text{ml}.$$

% Viability = viable cells per square/ total no. of cells per square x 100

$$= 40 / 44 \times 100 = 91\%$$

vi) The haemocytometer and cover slip was rinsed with 70% alcohol and wiped dry. Heterophil preparations obtained by this method were typically more than 98% pure and more than 95% viable.

vii) The cell concentration was adjusted to 1×10^7 heterophils/ml of RPMI 1640 for culture.

3.4: Culturing and stimulation of heterophils

The isolated Heterophils from six birds each from three breeds of birds, the broiler (B), the layer (L) and the Aseel (A) were randomly divided into three groups, each including six birds labeled alphanumerically (broiler into B0, B3, and B8, layer into L0, L3, and L8, further Aseel into A0, A3 and A8 respectively) on breed and time dependent manner, where alphabet represent breed and the number 0-hrs, 3-hrs, and 8- hrs represent the time period of LPS challenge in culture. Heterophils cultured in RPMI-1640 medium were stimulated with Lipopolysaccharide (LPS) of *Salmonella typhimurium* cell wall origin (L 2262, Sigma, St Louis, MO) with working concentration of 1µg / µl prepared in sterile tissue culture grade and endotoxin free water. The heterophil cultures 1ml per well were prepared in 6 well tissue culture plates and stimulates with 30 µg of LPS per ml of culture volume. The cultures were prepared near the flame in the laminar flow and incubated at 37º C at 5% CO_2 level under humidified conditions in a CO_2 incubator. Cultures were used for isolation of RNA at 0 hrs, after 3 hrs and after 8 hrs of LPS stimulation respectively.

3.5: Extraction of total RNA

The total RNA was isolated from heterophils at 0hrs, 3hrs and 8hrs of culture incubation using RNeasy mini kit (Qiagen) as per the instructed protocol:

1. Before using RPE concentrate provided with kit for the first time, 4 volumes of ethanol (96–100%) was added, as indicated on the bottle to obtain a working solution

2. 10 µl β- mercaptoethanol per 1 ml of RLT Buffer was added before use.
3. Cultured cells (1 x 10^7 cells) are pelleted by centrifuging for 5 min at 300 x g in a centrifuge tube. Supernatant was aspirated carefully.
4. Cells were disrupted using Buffer RLT. Pelleted cells were loosened by flicking the tube.
5. 700 µl of the sample was transfered, including any precipitate that was formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied with the kit). Lid was gently closed, and centrifuge for 15 s at 8000 x g . Flow-through was discarded.
6. 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuge for 15 s at 8000 x g to wash the spin column membrane. Flow-through was discarded.
7. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuge for 15 s at 8000 x g to wash the spin column membrane. Flow-through was discarded.
8. Again 500 µl Buffer RPE was added to the RNeasy spin column and centrifuge for 2 min at 8000 x g to wash the spin column membrane.
9. RNeasy spin column was placed in a new 1.5 ml collection tube. 30–50 µl RNase-free water added directly to the spin column membrane and centrifuge for 1 min at 8000 x g to elute the RNA.
10. Eluted RNA samples were treated with DNase (Qiagen RNeasy Mini Kit - RNA cleanup protocol).

3.6: Quantification and pooling of RNA

RNA concentration (quantification) and purity was checked using the spectrophotometer (Nanodrop 1000, Thermo Scientific) analysis using the convention

that 1 absorbance unit at 260 nm equals 40 µg RNA per ml. The U. V. absorbance checked at 260 and 280 nm for determination of sample concentration and purity. Purity of RNA judged on the basis of O.D. ratio at 260:280. The salt concentration as well as other impurities of RNA solution was judged on the basis of 230:260. The samples with acceptable purity (i.e. ratio 1.7-2.0) were further used in the study. Total RNA concentration was adjusted to 100 ng/µl using RNase free water. Equal amount of total RNA from six samples of same label were pooled.

3.7: First strand cDNA synthesis

Pooled total RNA was used for cDNA synthesis using First strand synthesis kit (Fermentas, Thermo scientific) using the following protocol:

1. 1 µl of olgo dT primer from the kit was added to 10 µl of 100ng/µl pooled RNA in a PCR tube and incubated at 65°C for 5 min and immediately chilled on ice.
2. Further the following components were added in the indicated order:

   5X Reaction Buffer : 4 µl

   RiboLock RNase Inhibitor (20 u/µl) : 1 µl

   10 mM dNTP Mix : 2 µl

   M-MuLV Reverse Transcriptase (20 u/µl) : 2 µl
3. Total volume of 20 µl reaction was incubated for 60 min at 37 °C.
4. To terminate the reaction the tubes were incubated for 5 min at 70 °C.

The reverse-transcription reactions were stored at -80°C for long term storage or used directly for polymerase chain reaction.

3.7.1: Confirmation of cDNA with GAPDH primers

The conversion to cDNA was checked by PCR with GAPDH primers provided with the kit (Forward primer: 5'-CAAGGTCATCCATGACAACTTTG-3' and Reverse primer: 5'-GTCCACCACCCTGTTGCTGTAG-3').The amplification of 496 bp GAPDH gene fragment from the cDNA indicated the presence of cDNA. PCR was carried out in a final reaction volume of 20 µl (Table 3.1).

Table 3.1: Components of PCR reaction mixture:

Sr. No.	Component	Concentration
1	10X PCR buffer (with $MgCl_2$ 15 mM)	2 µl
2	dNTPs (10 mM each)	1 µl
3	Forward GAPDH Primer (10pm/µl)	1 µl
4	Reverse GAPDH Primer (10pm/µl)	1 µl
5	Taq DNA polymerase (3 U/µl)	1 µl
6	cDNA Template (100ng/µl)	2 µl
7	Nuclease free water	12 µl
	Total	20 µl

Master mix was prepared for one additional sample to cover pipetting error. All reactions were carried out in 0.2 ml thin wall PCR tubes. PCR tubes containing mixture were tapped gently and quickly minispinned at 1000 rpm for few seconds. The tubes were placed in a thermal cycler and subjected to cycling reaction. The PCR protocol was same for all the primers (Table 3.2). The PCR reaction mix was subjected to 40 cycles of denaturation, annealing and extension as following:

Table 3.2: Cycling protocol for polymerase chain reaction

Sr. No.	Stage	Temperature (^{o}C)	Time
Initial Denaturation		94	3 min
1.	Denaturation	94	30 sec
2.	Annealing	58	30 sec
3.	Extension	72	45 sec
Repeat step 1-3 for 40 times			
Final extension		72	5.0 min.

3.7.2: Agarose gel electrophoresis of PCR product

To confirm PCR amplification, 5 µl of PCR product mixed with 1µl of 6X gel loading dye from each tube were electrophoresed on 2.0 % agarose gel (depending on the expected size of amplified product) containing 1% solution of ethidium bromide at the rate of 5µl/100 ml at constant voltage 85 V for 30 minutes in 0.5X TBE. The GeneRuler 1 kb Plus DNA Ladder (Fermentas) was also loaded to compare the size of the bands. The amplified product of 496 bp GAPDH gene was visualized as a compact band under UV light and documented by gel documentation system.

Table 3.3: Chemicals used for submarine gel electrophoresis

(A)	10X TBE , pH- 8.3	0.9M Tris HCl 0.9M Boric acid 20 MM EDTA
(B)	Gel loading dye (6X)	0.25% Bromophenol blue 0.25% Xylene cyanol FF 15% Ficoll Stored at room temperature
(C)	Ethidium bromide	5 µl (1%) per 100 ml agarose gel solution

3.8: Designing of primers for real-time PCR

Gene specific primers were designed from the respective gene sequences using online Primer3 software (http://frodo.wi. mit.edu/primer3/), such that the amplicon size were about 50 to 150 base pairs in length and that the annealing temperature of the reaction can be kept at 60 º C. β- actin primers were designed for internal control. These primers were custom synthesized by IDT (India).

The specificity of every pair of primer sequences was confirmed by BLASTn at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The amplified PCR fragments were checked for their size and specificity in 2.0% agarose gel electrophoresis.

The nomenclature and numbering system adopted for beta- defensin genes in the study is as used by Xiao *et al* (2004), as this system is currently used by the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/RefSeq/). Also this is in consistence with the usual nomenclature of other vertebrate beta-defensins. Therefore, term "avian beta-defensin" (abbreviated to AvBD) has been used, as proposed by Lynn *et al* (2007), instead of term gallinacin.

Table 3.4: The primer sequences, Genbank accession numbers of AvBD genes for relative quantitative real time PCR (Q- PCR) analysis are listed below:

Gene Name	Access No.	Primer Sequence (5' to 3')	Size (bp)
AvBD-1	AF033335	F CCTTGCTGTACCCTGAGAAACC	77
		R AGGTACACGATCCGCATGGT	
AvBD-2	AF033336	F CCAGGTTTCTCCAGGGTTGTC	65
		R GGCAGGACCCTCCTTTACAGA	

Gene Name	Access No.	Primer Sequence (5' to 3')	Size (bp)
AvBD-3	AF181952	F CTGTGGAAGAGCATATGAGGTTGAT	127
		R CACGGTCATACCATGGGAGACT	
AvBD-4	AY621306	F TTCTCTGCAGTGACAGGATTTCC	101
		R AAGCCCACAGCTCCATGAACT	
AvBD-5	AY621307	F CATGCAGATCCTGACTCTCCTCTT	131
		R GACATGACTTGTGGGAGCAGAA	
AvBD-6	AY621308	F CCAGCCCTATTCATGCTTGTAGA	121
		R CTGTTCCTCACACAGCAAGATTTTAG	
AvBD-7	AY621309	F TGCAGGTCAGCCCTTCATTC	121
		R GCCTATTCCATTGTTACATGTTCCA	
AvBD-8	AY621310	F TTGGCCGTTCTCCTCACTGT	137
		R TGCCCAAAGGCTCTGGTATG	
AvBD-9	AY621311	F GCCGTGCTCCTTCAGTTGA	67
		R GGTGCCCATTTGCAGCAT	
AvBD-10	AY621312	F CAAGATTCCGGCGCAGTAAG	74
		R CAAGGCAGTGGAAATGTTGCT	
AvBD-11	AY621313	F CTCTTCCTCCTCCAGGCTGTT	131
		R CAAGAGCATGTTCCAAATGCA	
AvBD-12	AY621314	F CCTTTGTTTCGTGTTCATCTTCATC	137
		R CAAAGCAGTACTTAGCCAGGTATTCC	
AvBD-13	AY621315	F GGAGGCTCTGCTTCCACATG	134
		R AAGGGTCCTGCTCTGCTGTGT	
AvBD-14	AM402954	F ATGGGCATATTCCTCCTGT	161
		R CACTTTGCCAGTCCATTGT	

Table 3.5: The primer sequences, Genbank accession numbers of chTLR genes for relative quantitative real time PCR (Q- PCR) analysis are listed below:

Gene Name	AccessNo.	Primer Sequence (5' to 3')	Size (bp)
chTLR 1	AB109401	F CCCAGAAGACTTGAGCGGAA	151
		R CCACGGCACATCCAGGTAG	
chTLR 2	AB046119	F GGCCTGAAAACCTGAAATATCTGA	79
		R TCAAGAGTTGAGGGAATGCAAGT	
chTLR 3	AY633575	F TTGACAGACTACCGGGAGTGTTT	94
		R TCCAGAGAGGTGAAGTTTGTCAAC	
chTLR 4	AY064697	F AGATGCAGAACCGAAGGCAA	76
		R TTGTGATGCTTTCCCACGTG	
chTLR 5	AY633576	F TGACATACGATGACTGCGATGA	84
		R TCAGAAGGGTGACAGATAGGAAAAC	
chTLR 7	AY633577	F TGACAACCTTTCCCAGAGCAT	91
		R TGTTGTTTTGAAAGTGCCACTTTTA	
chTLR 15	DQ267901	F TGCTGCCACATTTGGAAGATC	131
		R GATCGGTGCTCCACACAAGTC	
chTLR 21	AJ720600	F TCACAGGCGGAGGTCTTCAC	139
		R GCACCAACCCAGAGAAATCC	

Table 3.6: List of the primer sequences, Genbank accession numbers of Transcription Factor NFkB and IRF-3, cytokine IL-6 and internal control β -actin and 18s rRNA, for relative quantitative real time PCR (Q- PCR) analysis.

Gene Name	AccessNo.	Primer Sequence (5' to 3')	Size (bp)
β-actin	L08165	R AAGGGTCCTGCTCTGCTGTGT	78
		R TACCCAAGAAAGATGGCTGGAA	
18S rRNA	AF173612	F ATTGTGCCGCTAGAGGTGAAAT	71
		R CATTCTTGGCAAATGCTTTCG	
Interleukin-6 (IL-6)	NM_204628	F AGGACGAGATGTGCAAGAAGTTC	78
		R TTGGGCAGGTTGAGGTTGTT	
NFkB	D13719	F GAAGGAATCGTACCGGGAACA	131
		R CTCAGAGGGCCTTGTGACAGTAA	
IRF-3	U20338	F CCATCTTCGACTTCAGGGTGTT	134
		R CTTGGACTCCTTGGGCTTTGT	

3.9: Determination of primer/reaction efficiency

To estimate the efficiency of PCR reaction, 10-fold serial dilutions of cDNA was used in PCR reaction, starting with 10^7 template copies and ending with 10 copies. Template copies/molecules of DNA is calculated using:

$$\frac{\text{Mass (in grams)} \times \text{Avogadro's Number}}{\text{Average mol. wt. of a base} \times \text{template length}} = \text{molecules of DNA}$$

Average weight of bases is taken as 660 gm/mole/base was taken for double stranded DNA. Template length of the amplified product is used. Standard slope using log of template copies and Ct value was plotted to calculate the efficiency of the PCR reaction:

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1 * 100$$

3.10: Dose dependent expression of chTLR4 following 3 h incubation

Various dosage of LPS was tested for induction of chTLR4, which is the central component required by LPS as a signal transduction receptor. The heterophil cultures were stimulated with 10µg, 20µg, 30µg, 40µg and 50µg of LPS per ml of the culture volume and incubated for 3 hrs were used to study the relative expression of chTLR4 gene to study the induction in dose - dependent manner (Table 3.7):

Table 3.7: Preparation of a 6-well culture plate for LPS dose dependent expression study:

Treatment	Vol. of heterophils (µl)	Vol. of media (µl)	Vol. of LPS (µl)
Control	500	500	----
Treatment 1	500	490	10 (10µg)
Treatment 2	500	480	20 (20µg)
Treatment 3	500	470	30 (30µg)
Treatment 4	500	460	40 (40 µg)
Treatment 5	500	450	50 (50µg)

3.11: Relative gene expression

The cDNA was used to study the relative gene expression by gene specific amplification using Real Time PCR instrument (ABI prism 7500, Applied Biosystems) SYBR Green based chemistry. The reaction was carried out in a 96-well standard reaction plate, in the format as described in Table: 3.8.

Table 3.8: A sample 96- well standard RT-PCR reaction plate

	A	**B**	**C**	**D**	**E**	**F**	**G**
1	X Layer 0 hrs	X Layer 3 hrs	X Layer 8 hrs	β-actin 0 hrs	Y Layer 0 hrs	Y Layer 3 hrs	Y Layer 8 hrs
2	X Layer 0 hrs	X Layer 3 hrs	X Layer 8 hrs	β-actin 0 hrs	Y Layer 0 hrs	Y Layer 3 hrs	Y Layer 8 hrs
3	X Layer 0 hrs	X Layer 3 hrs	X Layer 8 hrs	β-actin 0 hrs	Y Layer 0 hrs	Y Layer 3 hrs	Y Layer 8 hrs
4	X Broiler 0 hrs	X Broiler 3 hrs	X Broiler 8 hrs	β-actin 3 hrs	Y Broiler 0 hrs	Y Broiler 3 hrs	Y Broiler 8 hrs
5	X Broiler 0 hrs	X Broiler 3 hrs	X Broiler 8 hrs	β-actin 3 hrs	Y Broiler 0 hrs	Y Broiler 3 hrs	Y Broiler 8 hrs
6	X Broiler 0 hrs	X Broiler 3 hrs	X Broiler 8 hrs	β-actin 3 hrs	Y Broiler 0 hrs	Y Broiler 3 hrs	Y Broiler 8 hrs
7	X Aseel 0 hrs	X Aseel 3 hrs	X Aseel 8 hrs	β-actin 8 hrs	Y Aseel 0 hrs	Y Aseel 3 hrs	Y Aseel 8 hrs
8	X Aseel 0 hrs	X Aseel 3 hrs	X Aseel 8 hrs	β-actin 8 hrs	Y Aseel 0 hrs	Y Aseel 3 hrs	Y Aseel 8 hrs
9	X Aseel 0 hrs	X Aseel 3 hrs	X Aseel 8 hrs	β-actin 8 hrs	Y Aseel 0 hrs	Y Aseel 3 hrs	Y Aseel 8 hrs
10	X NTC 0 hrs	X NTC 3 hrs	X NTC 8 hrs	β-actin NTC	Y NTC 0 hrs	Y NTC 3 hrs	Y NTC 8 hrs
11	X NTC 0 hrs	X NTC 3 hrs	X NTC 8 hrs	β-actin NTC	Y NTC 0 hrs	Y NTC 3 hrs	Y NTC 8 hrs
12	X NTC 0 hrs	X NTC 3 hrs	X NTC 8 hrs	β-actin NTC	Y NTC 0 hrs	Y NTC 3 hrs	Y NTC 8 hrs

Where, X and Y are target genes, NTC is No template control.

Reaction was carried out in a final reaction volume of 20 µL with standardized real time PCR reaction components (Table 3.9). The thermo- cycling parameters are given in Table 3.10. Nuclease free filter tips were used for taking the individual reaction components and preparation of reaction mixture. Careful pipetting was done without creating bubbles to avoid interference in reading of fluorescence by the instrument. No-template control (NTC) was put for either gene quantification or for checking the contamination in the reaction components other than the cDNA. These reagents were loaded on a real-time 96 wells optical plate (4306737, Applied biosystems, Foster city, CA) and sealed with optical adhesives cover (4313663, Applied biosystems, Foster city, CA) carefully without touching the optical portion of cover. To optimize the concentration of cDNA and primer, real time PCR was carried out with 2 fold serial dilution of cDNA and different primer concentration.

Table 3.9: Reaction mixture composition for q-PCR

2 x SYBR master mix	10 µl
Forward Primer (10 pm/ µl)	1 µl
Reverse Primer (10 pm/ µl)	1 µl
Nuclease free water	7 µl
cDNA (100ng/ µl)	1 µl
Total volume	20 µl

Table 3.10: Thermal cycler parameters for q-PCR

Stage	Temperature (°C)	Time	Activity
Stage 1	50	2 min	AmpErase UNG activation
Stage 2	95	10 min	AmpliTaq Gold DNA Pol. activation
Stage 3			
Step 1	95	15 sec.	Denaturation/ melting
Step 2	60	1 min.	Anneal/ extend

For Stage 3, steps 1 and 2, 40 cycles were performed.

Fluorescence was measured once every cycle after the extension step using filters for SYBR Green (excitation at 492 nm and emission at 530 nm) and the logarithm of the increment in fluorescence was plotted versus the cycle number. The threshold level was fixed at the same mid exponential position for all runs. At the end of extension step, fluorescent was captured. At the end of each run, a melt-curve analysis/dissociation curves (95^{o}C for 15 s, 60^{o}C for 1 min, and increase of 0.5^{o}C/5 s until 95^{o}C) was performed to assess the specificity of the amplification. The specificity of the amplification products were also confirmed by the appearance of predicted-size fragments after 2% agarose gel electrophoresis.

3.12: Data analysis

For quantification, data were analyzed with the Sequence Detection System (SDS) software v1.3.1 (Applied Biosystems). Comparative Ct method was used for relative quantification of the target gene relative to β-actin (endogenous control). The gene quantification has been expressed as ‘‘n-fold up/down regulation of transcription’’ in relation to an endogenous control. For relative quantification by the comparative Ct

method, values were expressed relative to a reference sample, called the calibrator. The Ct for the target gene and the Ct for the endogenous control were determined for each sample and calibrator.

The expression of selected gene was normalised by that of the reference gene, β-actin, at each time point and further converted to the fold increase (relative expression) over the calibrator, as follow:

$\Delta\Delta Ct = \Delta Ct$ sample A - ΔCt calibrator

Fold of Expression = $2^{-\Delta\Delta Ct}$

Where ΔCt is the average Ct of target gene - Average Ct of endogenous control (β-actin), $\Delta\Delta Ct$ is the average ΔCt of target sample - Average ΔCt of calibrator sample

Statistical analysis of normalized Ct value (ΔCt values) obtained from the quantitative PCR, from the three different chicken strains were subjected to analysis of variance (ANOVA) and there by post hoc Fisher's least significance difference test, for the significant values using SAS statistical software, Release 8.01 (SAS Institute, Inc Cary, NC). Statistical model used breed and time points as fixed effects. Differences were considered to be statistically significant at the level of $p < 0.05$.

CHAPTER IV

Results and Discussion

Selection of poultry for improved growth and production characteristics can adversely affect the ability of the bird to respond to pathogens and leave them more susceptible to infections and disease (Bayyari *et al* 1997). The indigenous breeds of chickens are generally considered to be more tolerant against a host of diseases than their improved commercial counterparts (Dhinkar *et al* 2009). Although, chicken strains show differences in susceptibility to a number of diseases (Zekarias *et al* 2002) the underlying immuno-genetic basis has not been elucidated. In the present investigation , innate immunity related, avian beta-defensin and toll-like receptor genes were evaluated for their relative expression with the stimulation of LPS at 0-hrs, 3-hrs, and 8-hrs time points in broiler, layer and indigenous Aseel.

4.1: Specificity of the primers

TaqMan probe and SYBR Green fluorescence reagents are currently most widely used in real-time PCR reaction for gene expression studies. Compared to TaqMan probe, SYBR Green is less expensive, but it requires additional procedures for checking the specificity of PCR amplification (Freeman *et al* 1999, Bustin 2002, Ponchel *et al* 2003). In this study, the primer's specificity was checked at three points: bioinformatics analysis, agar gel electrophoresis in regular PCR and dissociation curve analysis in real-time PCR. These measures guaranteed the specificity of PCR amplification in this research.

The specificity of each pair of primer sequences was confirmed by BLASTn at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The amplified PCR fragments were checked for their size and specificity in 2.0% agarose gel electrophoresis (Figure 4.1). Further derivative dissociation/melt curve generated for each PCR reaction showed single peak (Figure 4.2), justifying specific amplification. Each test PCR reaction was conducted in triplicate. Amplification plot (Figure 4.3) with cycle number on the horizontal axis, plotted against the relative fluorescence (Rn) on the vertical axis (log scale), when used for Ct values determination, showed very little variation in Ct value and lied within a range of 6 and 35 for all the reactions.

4.2: Screening of internal control

In relative quantitative RT-PCR analysis, the internal control (or endogenous control, reference or housekeeping gene) plays a critical role for checking the sensitivity, accuracy and reliability of this method. A good internal control should have stable expression and be independent of the impacts of test treatments or different tissue/cell types. Abundant expression should also be evaluated in the experimental design (Overbergh *et al* 2003). Ribosomal RNAs (18S or 28S rRNA in eukaryote and 16S rRNA in prokaryote, respectively) are widely used in real-time PCR experiments. Other stable expression genes include beta-actin and glyceraldehyde-3-phosphate dehydrogenase

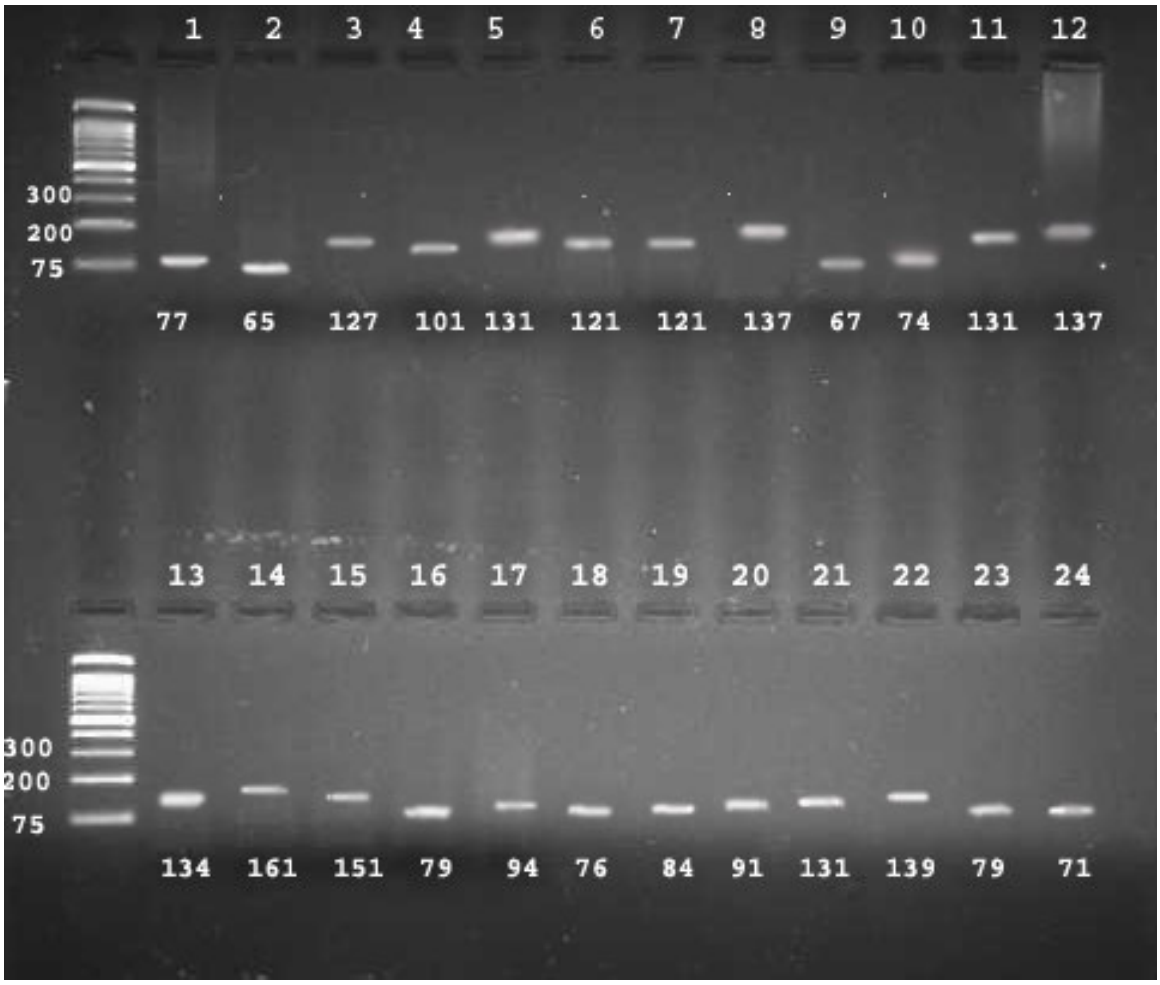

Figure 4.1: Gel electrophoresis pattern on 2% agarose, showing PCR amplification. First well in both rows show 1kb plus DNA ladder. Lane 1-14 show amplification of AvBDs; Lane 15-22 show amplification of chTLRs; Lane 23 and 24 show amplification of β – actin and 18S rRNA respectively.

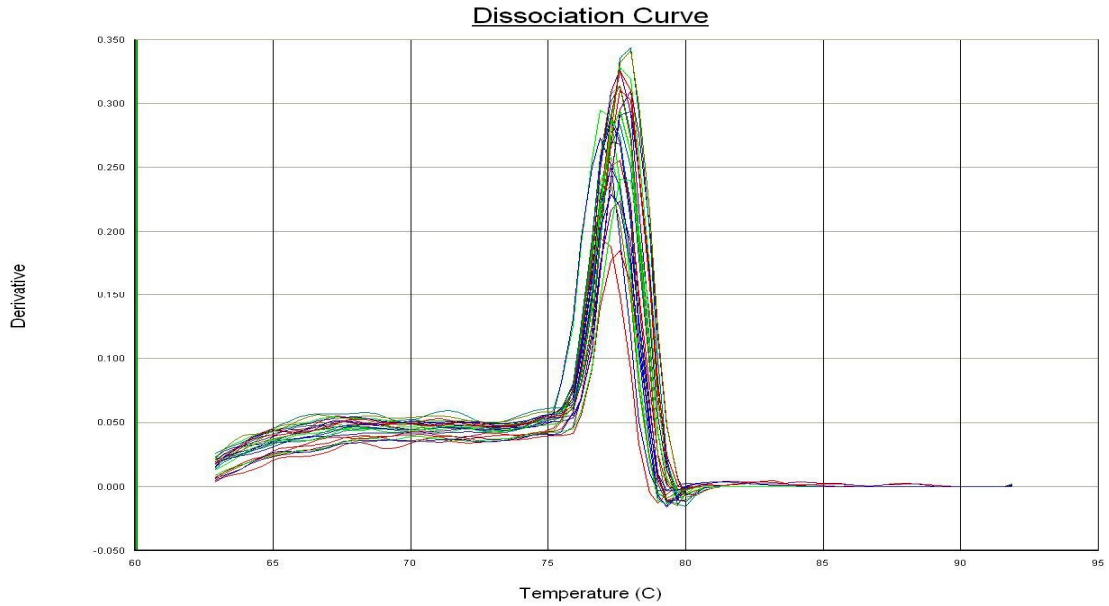

Figure 4.2: Melt-curve analysis/dissociation curves of β – actin gene (95^{o}C for 15 s, 60^{o}C for 1 min, and increase of 0.5^{o}C/5 s until 95^{o}C) to assess the specificity of the amplification.

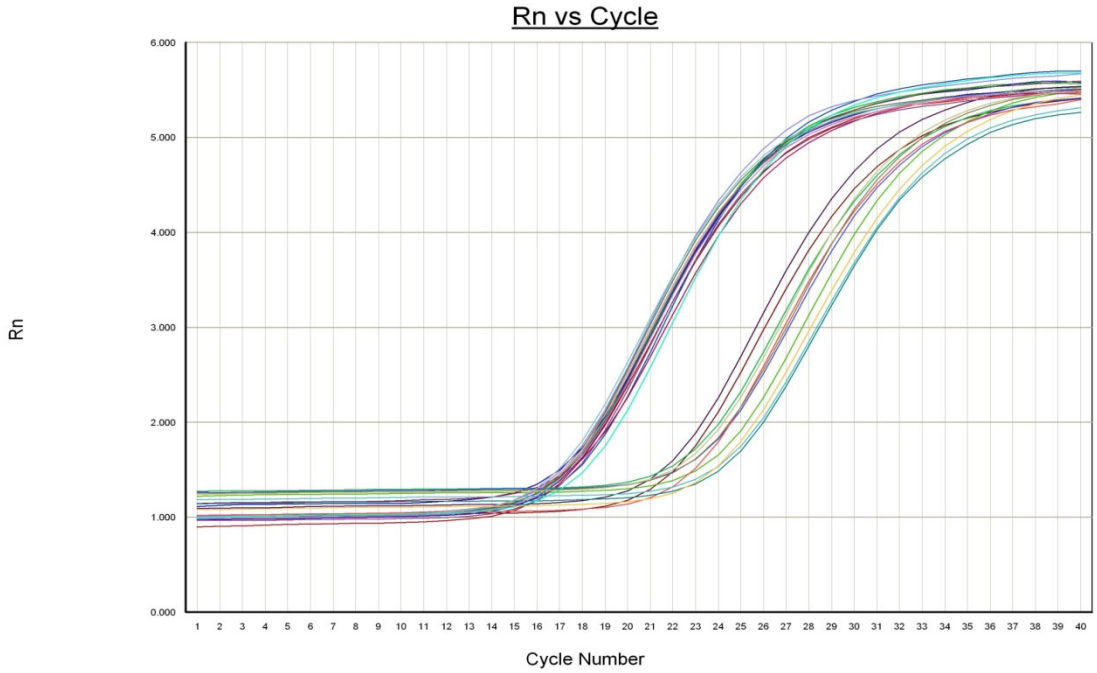

Figure 4.3: Quantitative-PCR amplification plots of the target genes and internal control gene, represented by fluorescence measured once every cycle after the extension step using filters for SYBR Green (excitation at 492 nm and emission at 530 nm) and the logarithm of the increment in fluorescence plotted versus the cycle number. The threshold levels were auto adjusted.

(GAPDH). However, the ribosomal RNAs sometimes do not truly reflect the overall RNA. Also, the beta-actin and GAPDH may have up-regulated expression in specific tissue or cell types such as the proliferating cells (Suzuki *et al* 2000). Combined internal controls or tissue- or cell-specific controls should be used in real-time PCR procedures (Vandesompele *et al* 2002 and Schmid *et al* 2003).

Therefore, two suitable internal controls were investigated in quantitative real-time PCR for stability of expression patterns in the present study, using a mixture of both random hexamer and oligo dT, during RT-PCR. The stability of two internal control genes (18S rRNA and beta-actin) were compared and the results from three independent experimental replicates are shown in Table. 4.1.

Table 4.1: Threshold cycle (Ct) of triplicate reactions for internal control genes in three independent replicates.

	Gene	**B0**	**B3**	**B8**	**L0**	**L3**	**L8**	**A0**	**A3**	**A8**
Rep I	β - actin	20.14	20.41	20.15	20.14	20.07	20.03	20.12	20.05	20.16
	18S rRNA	11.38	11.06	11.42	11.17	11.27	11.67	10.92	11.44	11.28
Rep II	β - actin	20.26	20.24	20.44	20.16	20.01	20.02	20.11	20.21	20.17
	18S rRNA	10.81	10.70	11.42	11.03	11.13	11.00	11.15	10.85	11.41
Rep III	β - actin	20.19	20.07	20.03	20.21	20.10	20.11	20.08	20.25	20. 19
	18S rRNA	11.12	11.37	11.87	11.79	11.20	11.23	11.75	11.56	11.51

Where , B0, B3, and B8 mean broiler at 0, 3 and 8 hrs time points respectively; L0, L3, and L8 mean layer at 0, 3, and 8 hrs time points respectively, and A0, A3, and A8 mean Aseel at 0, 3, and 8 hrs time points respectively.

The ranges of Ct value for beta-actin were 0.38 (replicate 1), 0.43 (replicate 2) and 0.22 (replicate 3), respectively. The corresponding ranges for 18S rRNA were 0.75 (replicate 1), 0.55 (replicate 2) and 0.67 (replicate 3), respectively. The comparison of the expression patterns of 18S rRNA and beta-actin revealed that beta-actin was more suitable to be used as internal control.

4.3: PCR efficiency

All PCR reactions displayed efficiency between 94 and 100 % (Table 4.2).

Table 4.2: Percentage efficiency (% E) of PCR reaction of target genes and internal controls.

Gene Name	% E	Gene Name	% E	Gene Name	% E
chTLR1	94	AvBD1	98	AvBD10	96
chTLR2	96	AvBD2	97	AvBD11	94
chTLR3	98	AvBD3	95	AvBD12	95
chTLR4	98	AvBD4	100	AvBD13	99
chTLR5	98	AvBD5	100	AvBD14	98
chTLR7	100	AvBD6	99	IL-6	99
chTLR15	98	AvBD7	99	NFkB	98
chTLR21	96	AvBD8	98	IRF-3	98
β -actin	100	AvBD9	95	18S rRNA	99

4.4: Dose dependent expressions of chTLR4

TLR-4 is a central component required by LPS as a signal transduction receptor (Gangloff and Gay 2004) and binding of LPS by the TLR-4 complex activated the signaling pathways that lead to increased gene expression (Froy 2005). Therefore, heterophil cultures stimulated with 10µg, 20µg, 30µg, 40µg and 50µg of LPS per ml of the culture volume and incubated for 3 hrs were used to study the relative expression of chTLR4 gene. A dose dependent pattern of expression was clearly evident (Figure 4.4). Using the expression of chTLR4 at 3hrs incubation with 10 µg/ml LPS dose, as calibrator, the relative expression was calculated and was found to be the highest for 20 µg/ml dose of LPS. The expression was 15 % higher (1.15 fold increase) in case of 20 µg/ml than 10 µg/ml dose, following which it declined for 30µg/ml antigen dose, and it drastically decreased on further increasing the dose. The plausible reason for this decrease might be tolerance induced by higher doses of LPS for the induction of TLR4, as was observed and suggested by Lorenzoni and Wideman (2008).

4.5: Expression profile of chTLR4 in heterophils

The expression of chTLR4 in broiler at 0-hrs, was taken as calibrator for calculation of relative expression/ fold change in broiler at other time points (3-hrs and 8-hrs) and also in calculation of fold change in layer and Aseel at all the time point (0-hrs, 3-hrs and 8-hrs) studied. The chTLR4 was induced in heterophils when stimulated with LPS, represented as fold-changes, calculated as $2^{-\Delta\Delta Ct}$ (Figure 4.5). As a most important PRR for LPS, chTLR4 had a highly up-regulated expression in all the genetic groups/strains at 3-hour time point (4.47 to 5.49 folds).

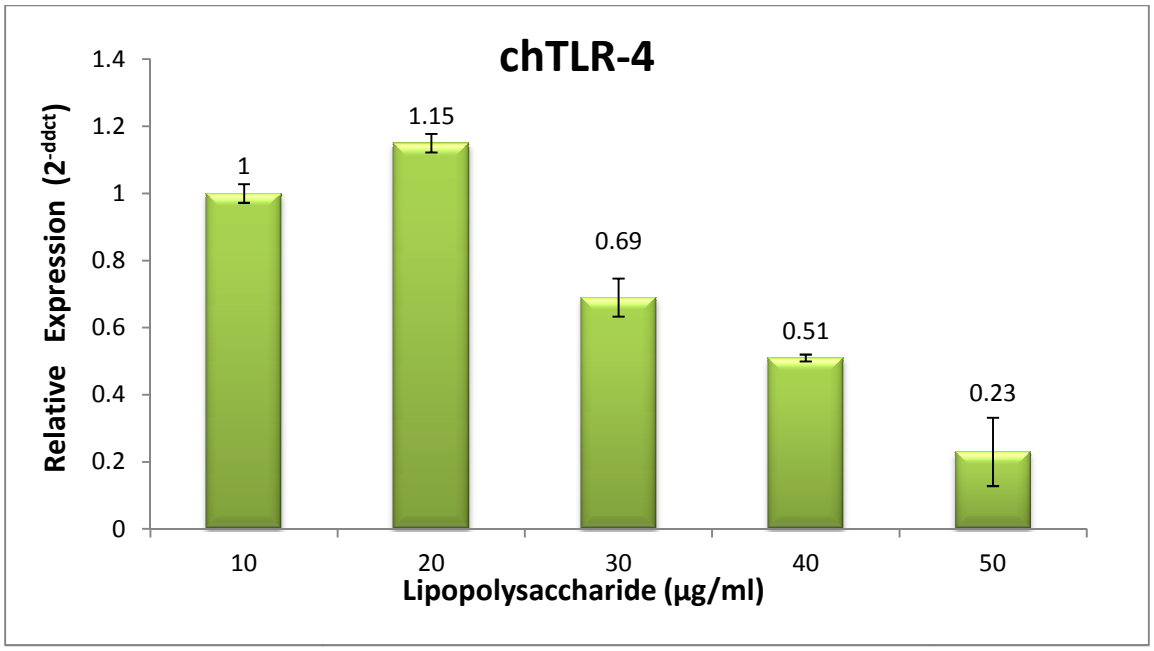

Figure 4.4: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of chTLR4 gene stimulated for 3 hrs using 10, 20, 30 40 and 50 µg/ml of LPS. Expression at 10 µg/ml dosage is taken as calibrator. β-actin was used as endogenous control.

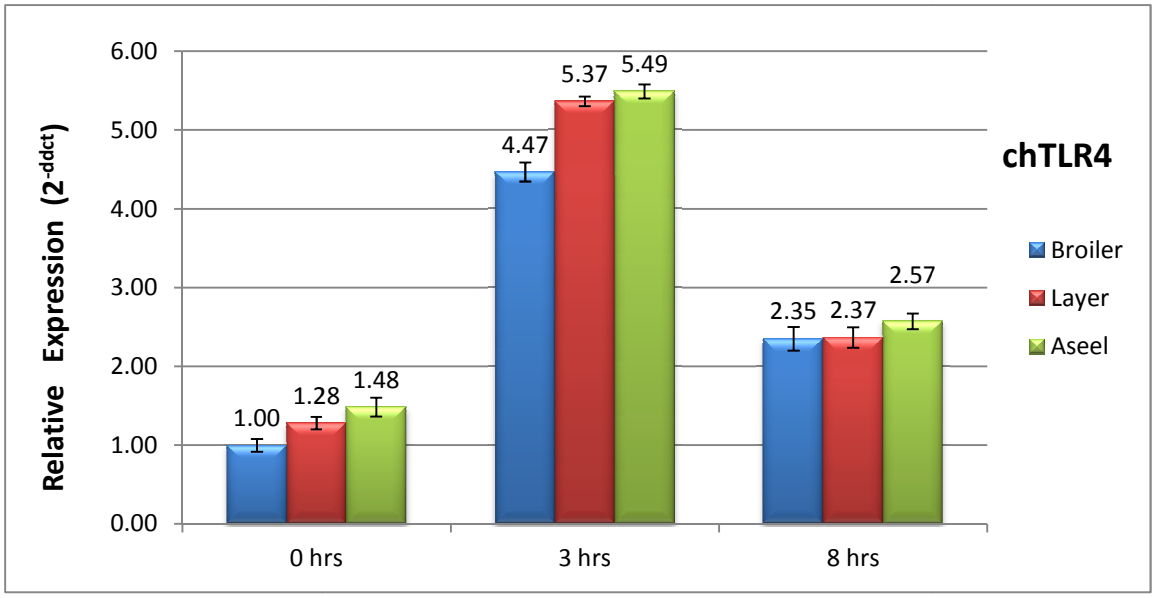

Figure 4.5: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of chTLR4 between broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. Expression of broiler at 0hrs is taken as calibrator. β-actin was used as endogenous control.

The expression reduced to 2.35 to 2.57 folds at 8-hrs time point, but, in general it was still up-regulated as compared to its initial expression, illustrating its function well beyond 8 hrs period.

LPS, as a powerful systemic stimulus, has been widely used to induce inflammation, although the efficacy of LPS can vary depending upon its origin, purity and dosage (Takahashi *et al* 1995, Sunwoo *et al* 1996 and Berczi 1998). The present study revealed significant increase ($p=0.000661$) of chTLR4 at 3 hrs in all the genetic groups. However, other chTLRs were not induced because the LPS used for the study was 99 % pure (S. enterica serovar typhimurium, L2262, Sigma-Aldrich).

Yang *et al* (1998) reported that TLR-2 could mediate cellular signaling when stimulated by LPS in human monocytes and macrophages. Kirschning *et al* (1998) also found TLR-2 mediated NFkB activation with LPS stimulation in human embryonic kidney 293 cells. However, the results from experiment on knockout mice revealed that TLR-4 deficient mice did not respond to LPS stimulation, whereas both TLR-2 deficient and wild-type mice responded to LPS stimulation with the same patterns (Takeuchi *et al* 1999). The impurities in LPS could be the reason of TLR-2 signaling pathway activation. After re-purification, it was confirmed that TLR-2 pathway was not activated in human by commercial LPS (Tapping *et al* 2000). The results of the present study uphold the thesis that chTLR2 or any other chTLRs are not induced by LPS.

4.6: Constitutive expression of chTLRs

The constitutive or the un-induced expression of all chTLR genes were detected in heterophils, and are represented as 40-ΔCt values (Figure 4.6). The chTLR4

constitutively expressed highest compared to other chTLRs, followed by chTLR5, 2, 3, 7, 15, 21 and 1 in order. This result corroborate with those of other workers (Iqbal *et al* 2005, Kogut *et al* 2005 and Musa *et al* 2009)

The constitutive expression of chTLR- 2, 3, 4, 7 and 15 was significantly higher expression in layer compared to broiler stock (Table 4.3). chTLR 1 and 7 expression was significantly higher in Aseel, compared to layer. All chTLRs expressed significantly higher in Aseel with an exception of, chTLR 5 and 21 as compared to broiler. This ubiquitous expression profile of chTLRs illustrates their important role in pathogen recognition during avian innate immune response, where heterophils are important effector cells.

Table 4.3: Relative Expression levels (ΔΔCt) of chTLRs in broiler, layer and Aseel without LPS stimulation. Negative values indicate relatively higher expression.

Gene Name	**I**	**II**	**III**
	Broiler vs Layer	**Layer vs Aseel**	**Broiler vs Aseel**
chTLR1	-0.1467 (p=0.155)	**-1.8447** (p=0.134)	**-1.9914** (p=0.042)
chTLR2	**-1.8535** (p=0.001)	-0.1236 (p=0.549)	**-1.9771** (p=0.016)
chTLR3	**-1.2318** (p=0.016)	-0.5863 (p=0.425)	**-1.8181** (p=0.022)
chTLR4	**-1.2381** (p=0.024)	-0.4981 (p=0.355)	**-1.7362** (p=0.012)
chTLR5	-0.3761 (p=0.330)	-0.5582 (p=0.200)	-0.9343 (p=0.365)
chTLR7	**-1.2416** (p=0.049)	**-1.0317** (p=0.035)	**-2.2733** (p=0.020)
chTLR15	**-1.4321** (p=0.012)	-0.1688 (p=0.325)	**-1.6009** (p=0.013)
chTLR21	-0.4942 (p=0.170)	0.5482 (p=0.443)	0.0540 (p=0.677)

* bold values indicate significant difference in target gene expression (P<0.05)

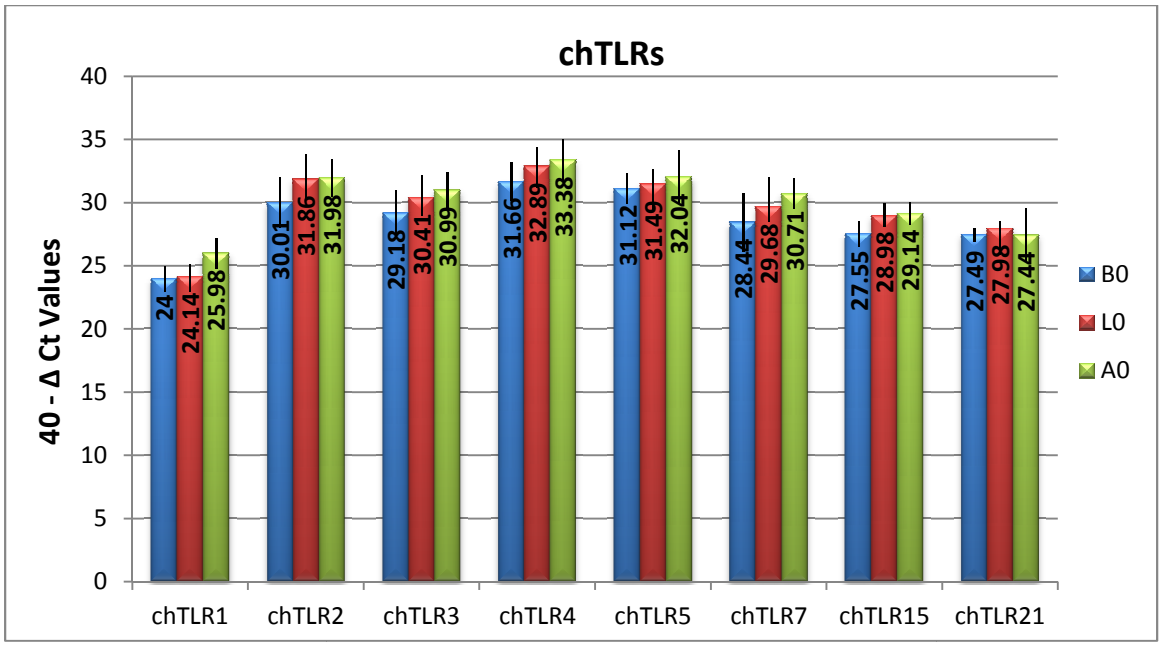

Figure 4.6: 40- ΔCt values of chTLRs in broiler, layer and Aseel without LPS stimulation. β-actin was used as endogenous control.

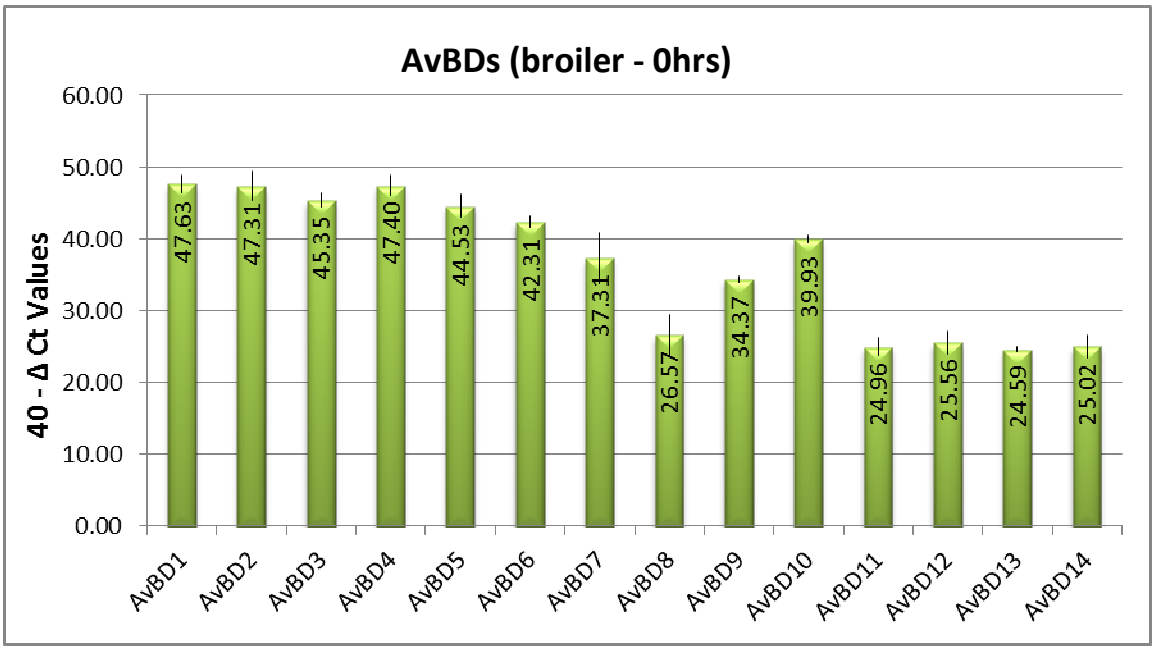

Figure 4.7: 40- ΔCt values of AvBDs (broiler-0hrs) in broiler, layer and Aseel without LPS stimulation. β-actin was used as endogenous control.

4.7: Expression profile of AvBDs in heterophils.

General trend of expression: Taking into account the gene expression of all the three stains (broiler, layer and Aseel) together the expression analysis revealed that all the 14 AvBD genes expression were detectable in heterophils. The expression of AvBDs in broiler at 0-hrs, which was taken as calibrator for further calculation of relative expression/ fold change, was plotted as 40-ΔCt values (Figure. 4.7), to understand the constitutive expression pattern of various AvBD genes in heterophils.

AvBD-1, 2 and 4 were among the high expressing genes in blood heterophils. While, AvBD- 8, 11, 12, 13 and 14 were among the low expressing genes across all the three strains of birds, but only the data for broiler are presented, as it has been used as a calibrator for calculation of relative expression/fold change in other strains.

Further, the expression levels of all AvBDs at different time points (0 hrs, 3 hrs and 8hrs) with the stimulation of LPS were investigated. The expression of AvBDs in broiler at 0-hrs time point, was used as calibrator for the calculation of relative expression/ fold change in broiler at other time points (3-hrs and 8-hrs) and also in calculation of fold change in layer and Aseel at all the time point (0-hrs, 3-hrs and 8-hrs) studied and presented as fold-changes, calculated as $2^{-\Delta\Delta Ct}$ (Figure 4.8 to Figure 4.21).

At early phase (at 3 hrs period) most of the 14 AvBD genes studied showed up-regulation in response to LPS at early phase i.e. within 0-3 hrs period, however the up-regulation in AvBD- 9,11,12,13 and 14 was not significant.

At late phase, that is 8-hrs after LPS stimulation, the expressions of most AvBDs were down-regulated to counteract the effects of the increased transcription in the early

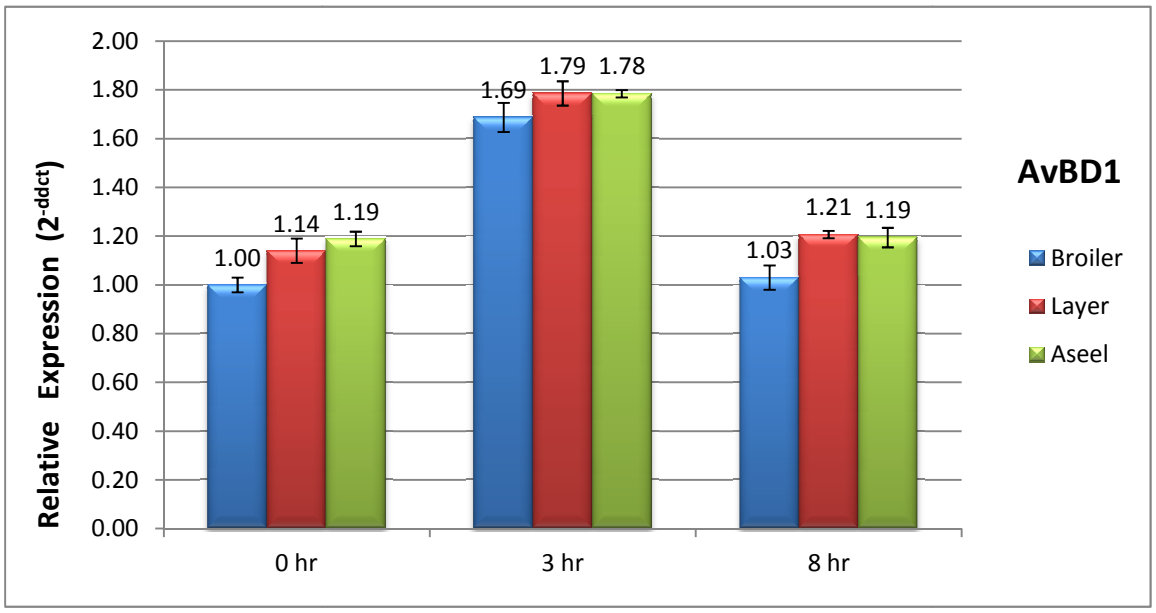

Figure 4.8: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD1 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

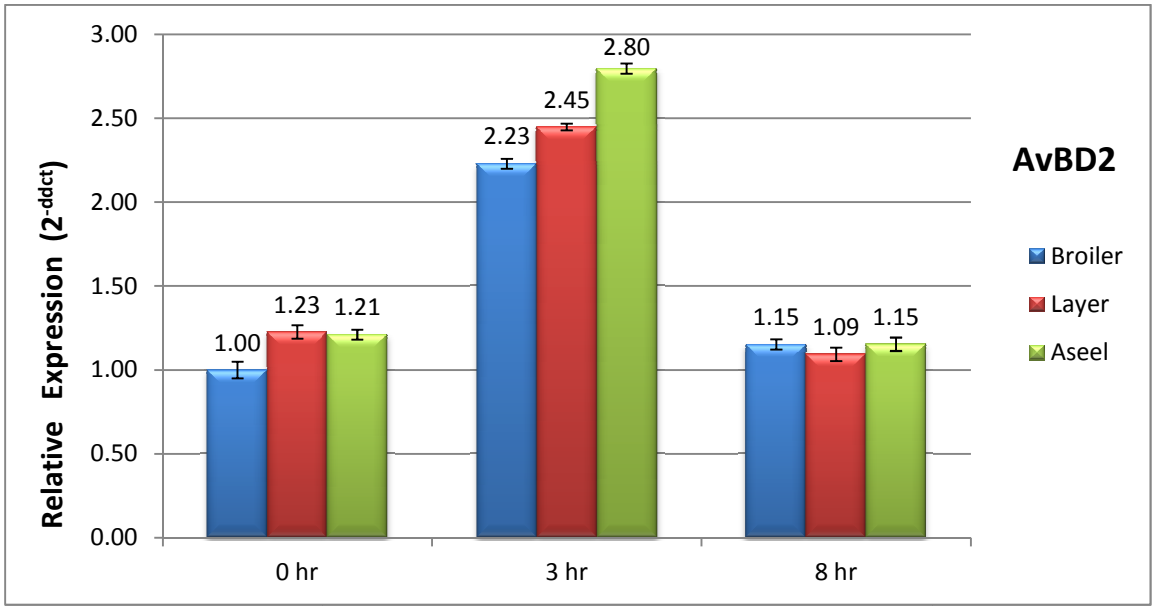

Figure 4.9: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD2 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

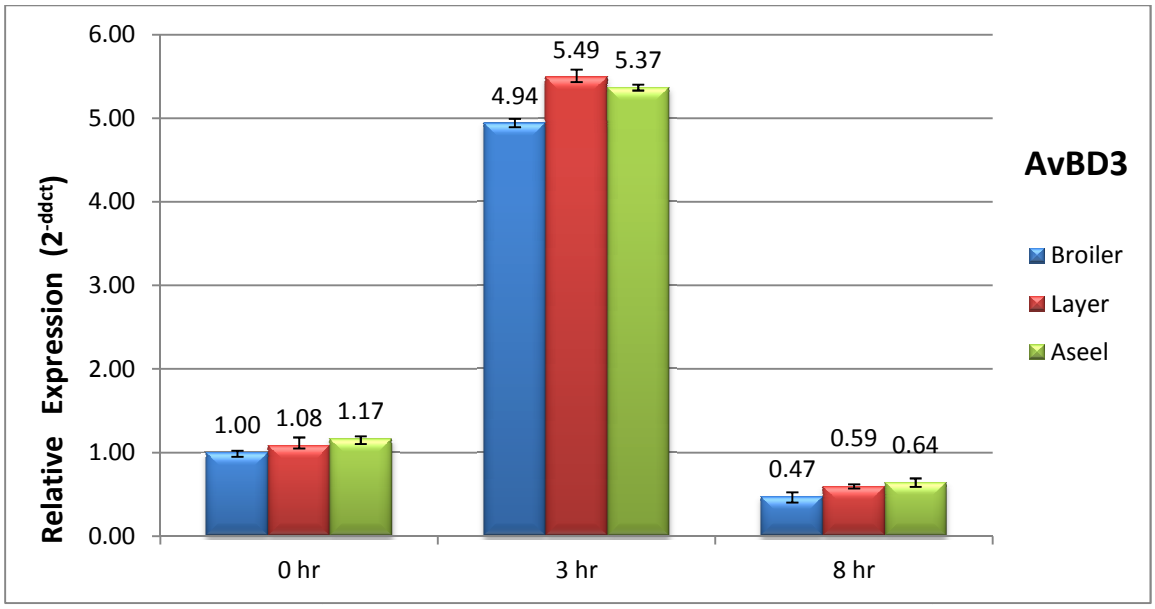

Figure 4.10: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD3 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

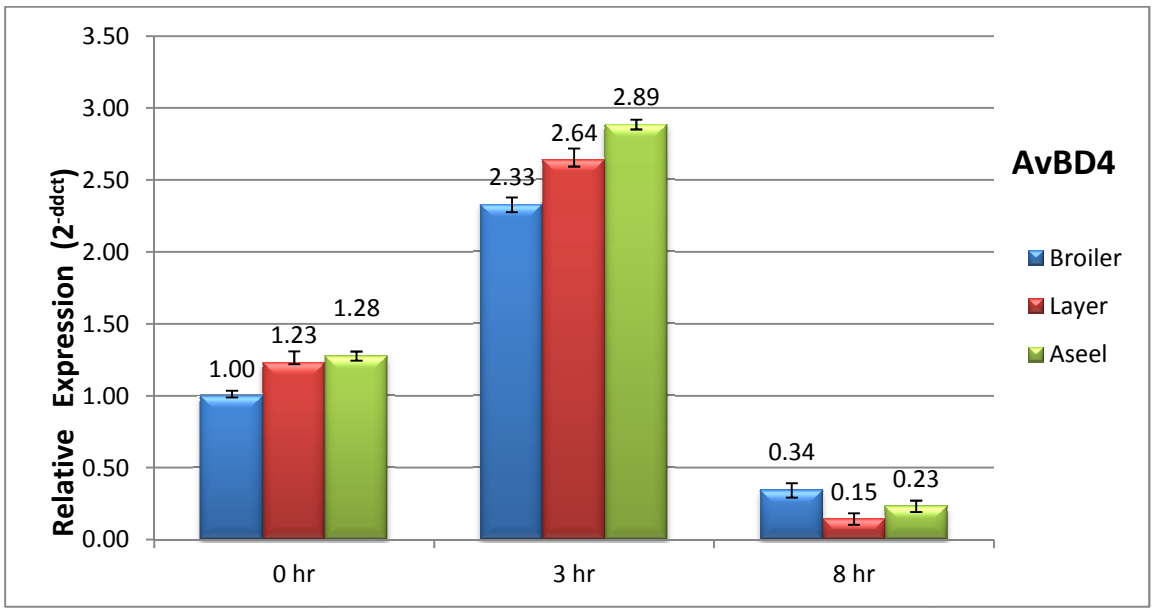

Figure 4.11: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD4 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

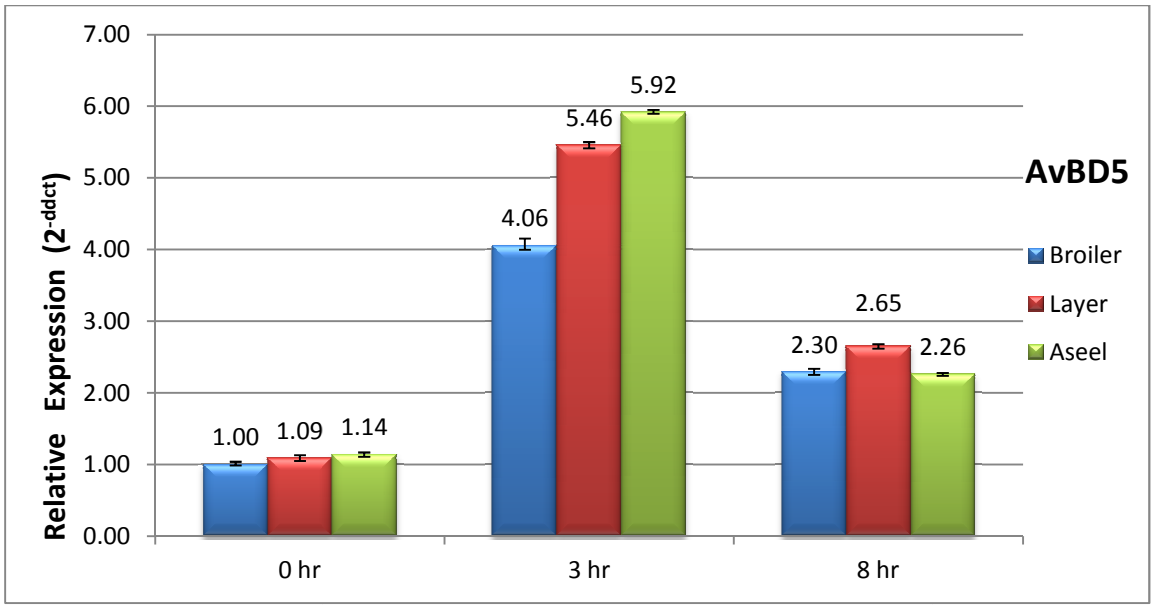

Figure 4.12: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD5 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

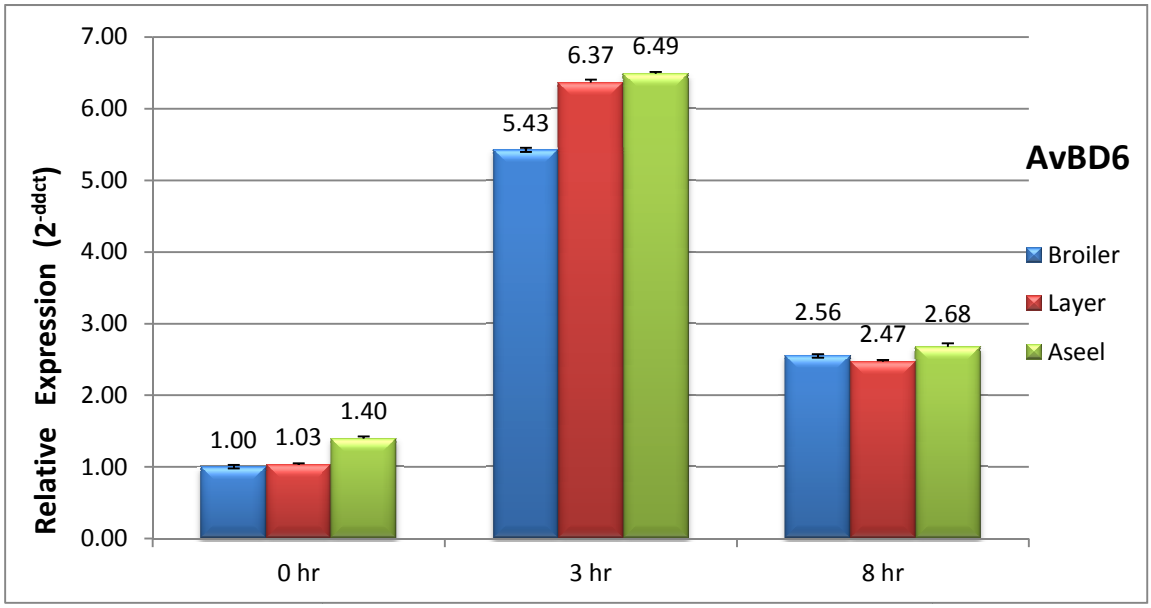

Figure 4.13: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD6 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

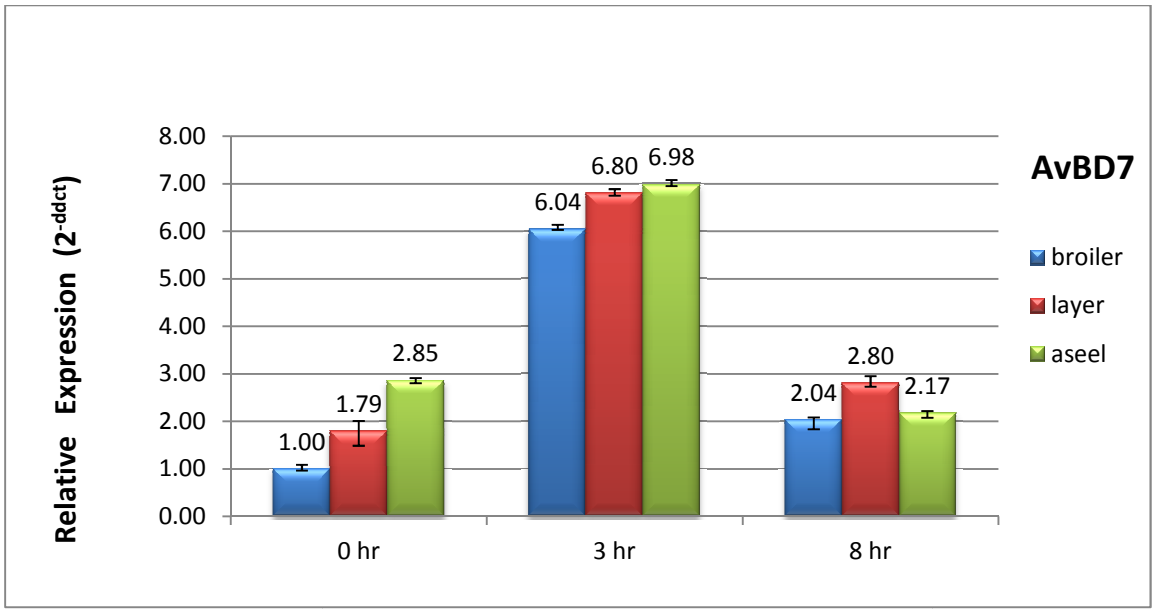

Figure 4.14: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD7 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

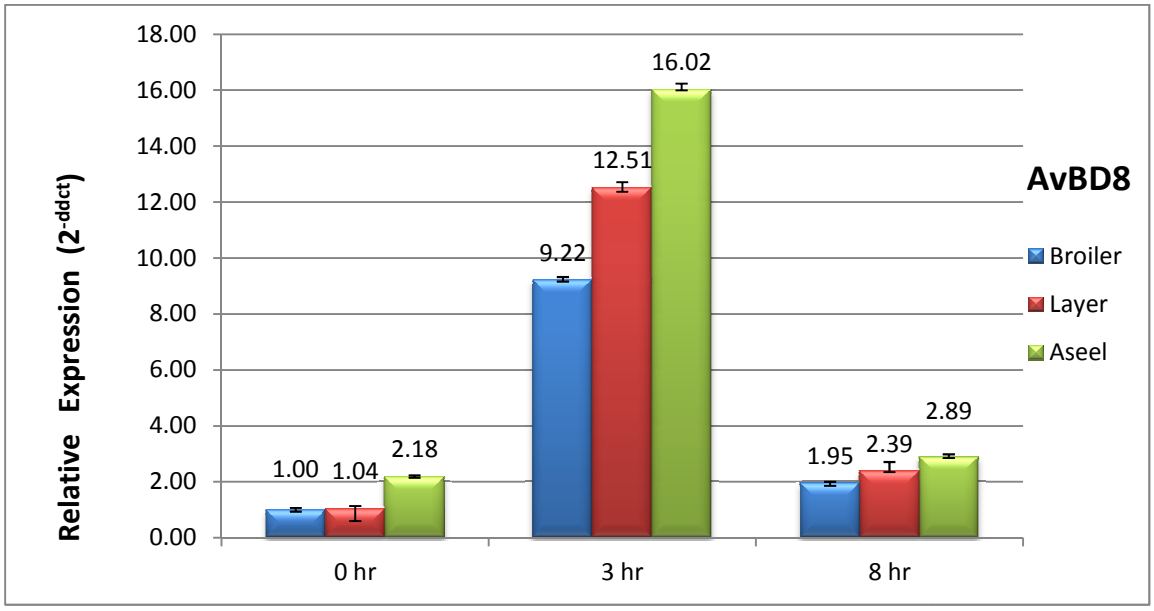

Figure 4.15: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD8 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

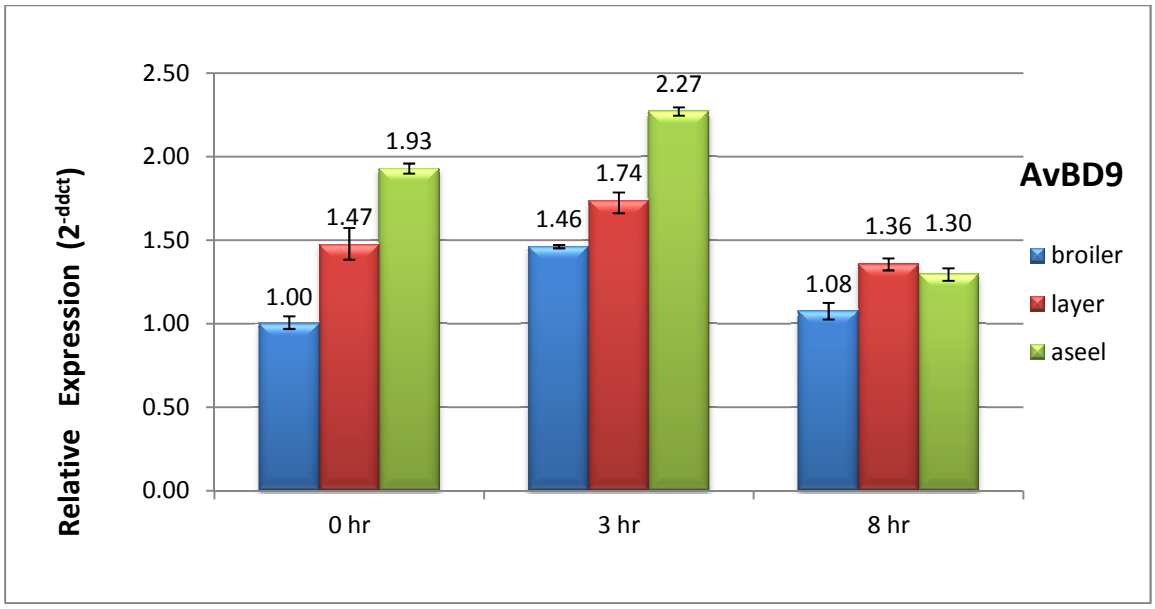

Figure 4.16: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD9 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

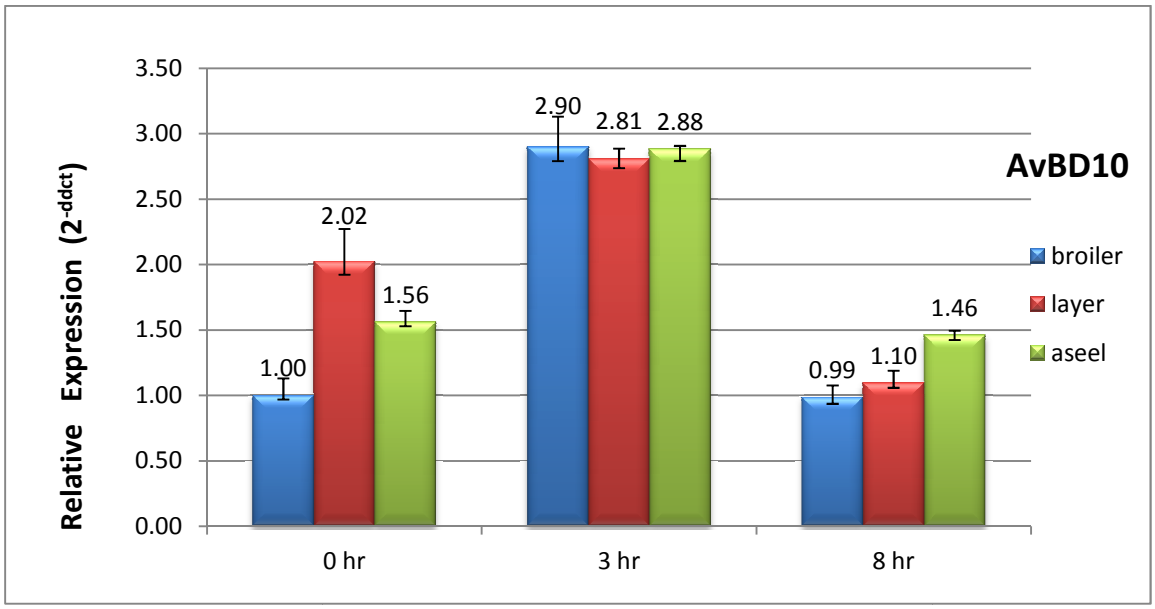

Figure 4.17: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD10 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

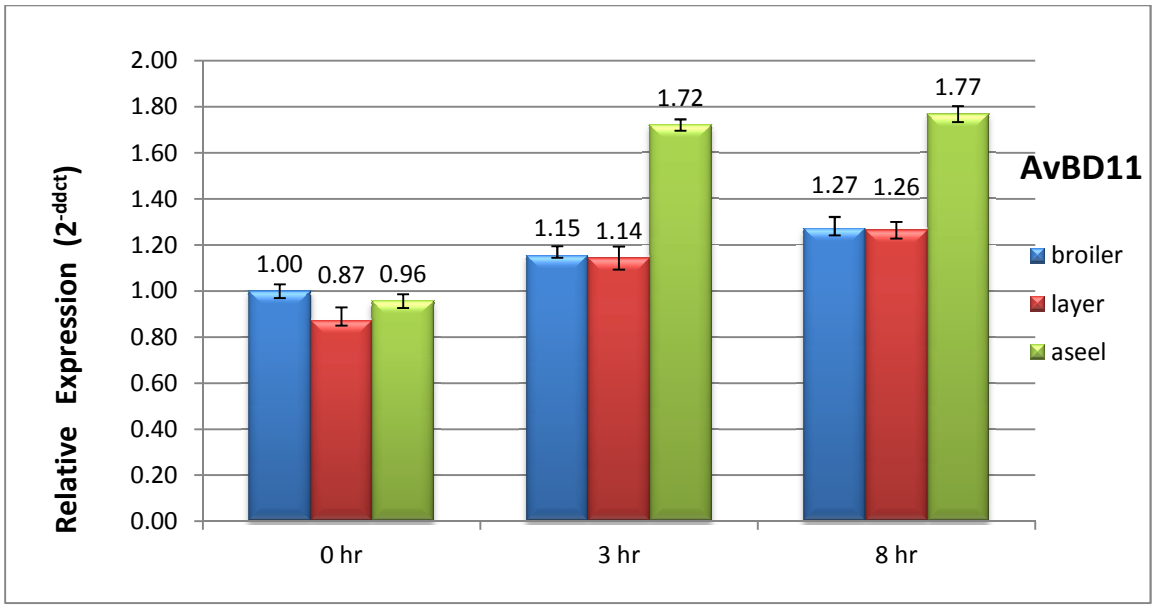

Figure 4.18: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD11 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

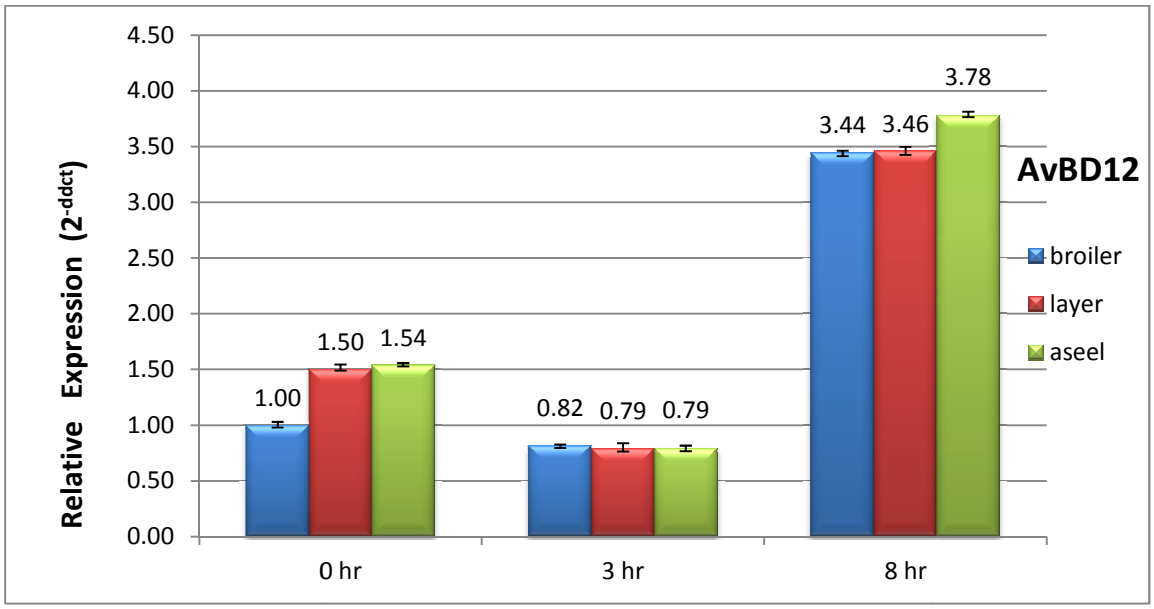

Figure 4.19: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD12 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

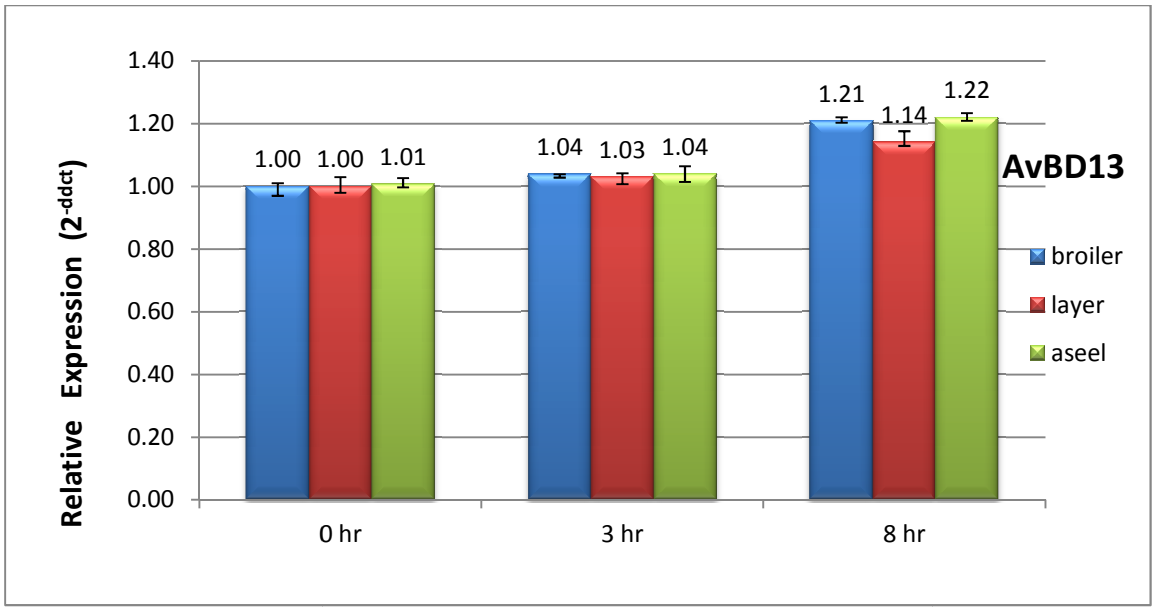

Figure 4.20: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD13 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

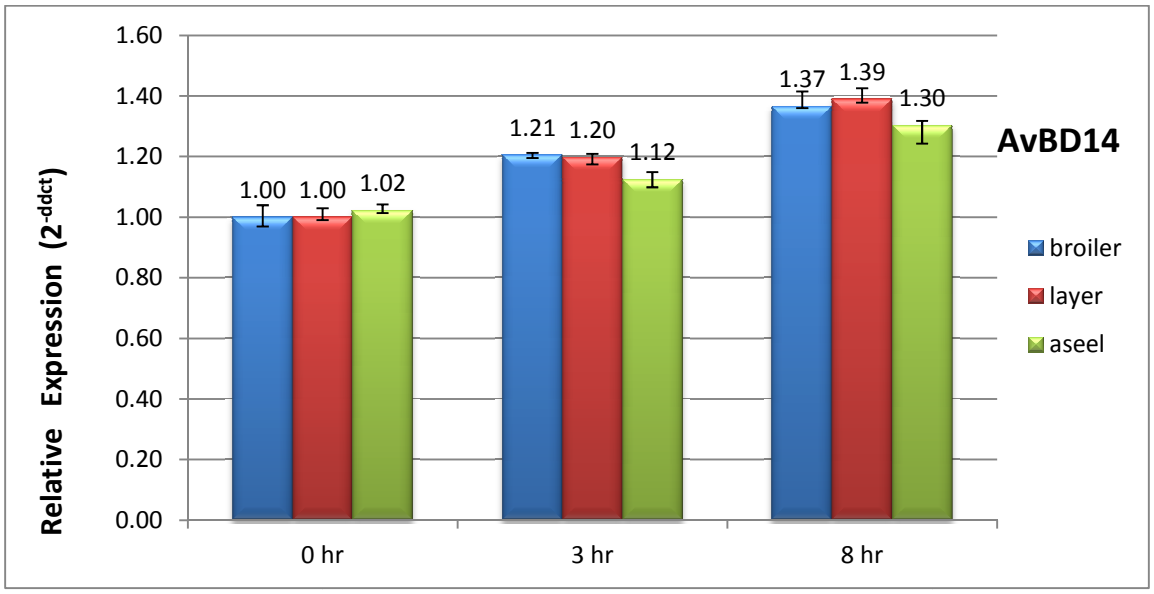

Figure 4.21: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of AvBD14 in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

phase. This counteraction might occur because the host needs to maintain the inner homeostasis via a negative feedback mechanism to counteract the increased transcription level.

Before LPS stimulation, the relative gene expression of AvBD- 8, 11, 12,13 and 14 expressed at low level in the blood heterophils, out of which AvBD8 was maximally induced by LPS at 3 hrs (up to 9.22 to 16.02 fold increase) at early phase and maximally down-regulated to (1.95 to 2.89 folds) at later phase. Whereas other, early phase low expressing genes AvBD- 11, 12, 13, and 14 did not show sharp up-regulation at early phase of LPS stimulation, but continued to up-regulated in late phase i.e. even after 3 hrs period. AvBD- 1, 2 and 4 gene expression was in the highest in blood, but the up-regulation on induction was only 1.68 folds to 2.88 folds in the early phase, maybe because they already expressed at a high level in the blood at the start. AvBD12 had lower expressions (0.78 to 0.81 folds) and AvBD13 was not induced, with the challenge of LPS at early phase.

When compared together, genes showing high constitutive expression showed lesser induction by LPS and the genes showing low constitutive expression either showed sharp early phase induction or continued to express for a longer time period (upto 8 hrs tested). At the later phase (from 3- to 8-hrs), most AvBDs showed reduced expression, possibly to counteract the effects of increased transcription at the early phase, but the overall effect (from 0- to 8-hrs) of LPS stimulation can still be regarded as induction for most AvBDs, except for AvBD 3, 4 and 7, where the expression at 8 hrs was found to be less than at 0 hrs.

4.8: Differential gene expression of AvBDs in broiler, layer and Aseel birds.

Constitutive Expression: When the constitutive expression of AvBD genes was compared in broiler, layer and Aseel, the expression of almost all the genes was higher in layer compared to broiler, and in Aseel compared to layer (Table 4.4).

Differential expression calculated as ΔΔCt values indicated that, although expression almost all the AvBDs was higher in layer compared to broiler (indicated by negative values) but not in case of AvBD- 11, 13, 14. However the difference between the layer and broiler was statistically significant in layer compared to broiler only in case of AvBD- 2, 4, 7, 9 and 10. The constitutive expression of AvBD- 6, 7, 8, 9 and 11 in Aseel was significantly higher than layer, with the only exception of AvBD10. When broiler and Aseel are compared most of the AvBD expression was significantly higher in Aseel, except AvBD- 5, 11, 12, 13 and 14.

Early phase: AvBD- 5, 6, 8 and 9 expressed significantly higher in layer than broiler at early phase (quantified at 3hrs time point). However, the expression of AvBD- 8, 9 and 11 was significantly higher in Aseel compared to layer. The expression of AvBD- 2, 4, 5, 6, 7, 8, 9 and 11 was significantly higher in Aseel compared to broiler at early phase.

Late Phase: Six out of fourteen AvBDs (AvBD- 1, 3, 5, 7, 8 and 9) were significantly up-regulated in layer compared to broiler. However, in Aseel strain four AvBDs (AvBD- 4, 8, 10 and 11) were significantly up-regulated compared to layer, and six AvBDs (AvBD- 1, 3, 8, 9, 10 and 11) were significantly up-regulated compared to broiler.

4.8.1: Differential expression between the strains

The expression of most AvBDs was higher in layer compared to broiler (Table 4.4), but the differential expression between layer and broiler was statistically significant (negative values indicate higher expression in layer) for AvBD-1 at 8 hrs, AvBD-2 at 3 hrs, AvBD-3 at 8 hrs, AvBD-5 at 3 hrs and 8 hrs, AvBD-6 at 3 hrs, AvBD-7 at 0 hrs and 8 hrs, AvBD-8 at 3 hrs and 8 hrs, AvBD-9 at all three time points (0 hr, 3 hr and 8 hr), AvBD-10 at 0 hrs, whereas AvBD-11 expression was significantly down-regulated at 0 hrs in layer (indicated by positive value).

Similarly, though the Aseel had higher level of expression compared to layer, for most of the AvBD genes, but the difference was significantly higher for AvBD-4 at 8 hrs, AvBD-6 and 7 at 0 hrs, AvBD-8 at 0 hrs, 3 hrs and 8 hrs, AvBD-9 at 0 hrs and 3 hrs, AvBD-10 at 8hrs, AvBD-11 at 0 hrs, 3 hrs and 8 hrs. While the expression of AvBD-5 and 8, at 8 hrs, AvBD-10 at 0 hrs was significantly lower in Aseel compared to layer.

When Aseel and broiler were compared, the expression of AvBD-1 at 0 hrs and 3 hrs, AvBD-2 at 0 hrs and 3 hrs, AvBD-3 at 0 hrs and 8 hrs, AvBD-4 at 0 hrs and 3 hrs, AvBD-5 at 3 hrs, AvBD-6 and 7 at 0 hrs and 3 hrs, AvBD-8 and 9 at 0 hrs, 3 hrs and 8 hrs, AvBD-10 at 0 hrs and 8 hrs, AvBD-11 at 3 hrs and 8 hrs was significantly higher in Aseel compared to broiler. However the expression of AvBD-4 at 8 hrs was significantly lower in Aseel compared to broiler. Expression of AvBDs in heterophils have not been studied before.

Table 4.4: Relative Expression levels (ΔΔCt) of AvBDs in broiler, layer and Aseel. Negative values indicate relatively higher expression.

Gene Name	I	II	III
AvBD-1	**Broiler vs Layer**	**Layer vs Aseel**	**Broiler vs Aseel**
0hr	-0.1896 (p= 0.666)	-0.0599 (p=0.071)	**-0.2496** (p=0.017)
3hr	-0.0818 (p=0.415)	0.0008 (p=0.016)	-0.0809 (p=0. 695)
8hr	**-0.2286** (p=0.026)	0.0149 (p=0.307)	**-0.2137** (p=0. 942)
AvBD-2			
0hr	**-0.2947** (p=4E-04)	0.0189 (p=0.003)	**-0.2757** (p=0. 041)
3hr	-0.1355 (p=0.089)	-0.1914 (p=0.521)	**-0.3269** (p=0.035)
8hr	0.0750 (p=0.808)	-0.0761 (p=0.805)	-0.0011 (p=0.997)
AvBD-3			
0hr	-0.1144 (p=0.593)	-0.1060 (p=0.055)	**-0.2204** (p=0.015)
3hr	-0.1524 (p=0.478)	0.0336 (p=0.874)	-0.1188 (p=0.580)
8hr	**-0.3262** (p=6E-05)	-0.1177 (p=0.073)	**-0.4439** (p=1E-06)
AvBD-4			
0hr	**-0.2996** (p=0.023)	-0.0514 (p=0.836)	**-0.3510** (p=0.017)
3hr	-0.1809 (p=0.470)	-0.1284 (p=0.771)	**-0.3093** (p=0.006)
8hr	1.2445 (p=0.239)	**-0.6819** (p=9E-05)	**0.5625** (p=0.030)
AvBD-5			
0hr	-0.1246 (p=0.721)	-0.0637 (p=0.855)	-0.1883 (p=0. 591)
3hr	**-0.4283** (p=0.002)	-0.1170 (p=0. 738)	**-0.5453** (p=0.001)
8hr	**-0.2076** (p=0.006)	**0.2314** (p=0.048)	0.0238 (p=0.891)
AvBD-6			
0hr	-0.0461 (p=0.730)	**-0.4345** (p=0.004)	**-0.4806** (p=0.002)
3hr	**-0.2299** (p=0.008)	-0.0278 (p=0.835)	**-0.2577** (p=0.020)
8hr	0.0494 (p=0.071)	-0.1157 (p=0.392)	-0.0663 (p=0.621)
AvBD-7			
0hr	**-0.8400** (p=0.003)	**-0.6733** (p=7E-04)	**-1.5133** (p=6E-5)
3hr	-0.1712 (p=0.111)	-0.0376 (p=0.668)	**-0.2089** (p=0.002)
8hr	**-0.4606** (p=0.007)	**0.3682** (p=0.001)	-0.0923 (p=0.229)
AvBD-8			
0hr	-0.0515 (p=0.739)	**-1.0736** (p=0.004)	**-1.1251** (p=0.004)
3hr	**-0.4403** (p=0.001)	**-0.3565** (p=0.003)	**-0.7968** (p=0.003)
8hr	**-0.2919** (p=0.003)	**-0.2723** (p=0.021)	**-0.5642** (p=0.001)

AvBD-9			
0hr	**-0.5601** (p=0.006)	**-0.3886** (p=0.004)	**-0.9486** (p=0.004)
3hr	**-0.2484** (p=0.049)	**-0.3872** (p=0.037)	**-0.6357** (p=0.008)
8hr	**-0.3332** (p=0.017)	0.0641 (p=0.821)	**-0.2691** (p=0.001)
AvBD-10			
0hr	**-1.0172** (p=0.015)	**0.3755** (p=0.048)	**-0.6416** (p=0.001)
3hr	0.0468 (p=0.084)	-0.0377 (p=0.906)	0.0090 (p=0.977)
8hr	-0.1510 (p=0.638)	**-0.4145** (p=0.019)	**-0.5655** (p=0.007)
AvBD-11			
0hr	**0.5228** (p=0.041)	**-0.4946** (p=0.004)	0.0281 (p=0.964)
3hr	0.0142 (p=0.982)	**-0.5902** (p=0.003)	**-0.5760** (p=0.042)
8hr	0.0091 (p=0.988)	**-0.4846** (p=0.003)	**-0.4755** (p=0.044)
AvBD-12			
0hr	-0.0244 (p=0.809)	-0.0364 (p=0.719)	-0.0608 (p=0.549)
3hr	0.0509 (p=0.614)	-0.0071 (p=0.944)	0.0438 (p=0.663)
8hr	-0.0079 (p=0.937)	-0.1262 (p=0.195)	-0.1341 (p=0.221)
AvBD-13			
0hr	0.0002 (p=0.999)	-0.0102 (p=0.917)	-0.0100 (p=0.919)
3hr	0.0112 (p=0.739)	-0.0125 (p=0.899)	-0.0013 (p=0.645)
8hr	0.0839 (p=0.187)	-0.0965 (p=0.399)	-0.0126 (p=0.334)
AvBD-14			
0hr	0.0003 (p=0. 997)	-0.0325 (p=0.713)	-0.0322 (p=0.716)
3hr	0.0097 (p=0.912)	0.0916 (p=0.307)	0.1013 (p=0.260)
8hr	-0.0253 (p=0.774)	0.0925 (p=0.302)	0.0672 (p=0.450)

* bold values indicate significant difference in target gene expression (P<0.05)

4.9: Differential gene expression NFkB-1 and IRF-3

In the present study, two important transcription factors (NFkB-1 and IRF-3) were investigated for differential expression at 0 hrs, 3 hrs and 8 hrs of LPS stimulation (Figure. 4.22). The results from this study revealed that both NFkB-1 and IRF-3 were induced early phase (3hrs time point) and the expression decreased at later phase (8hrs time point).

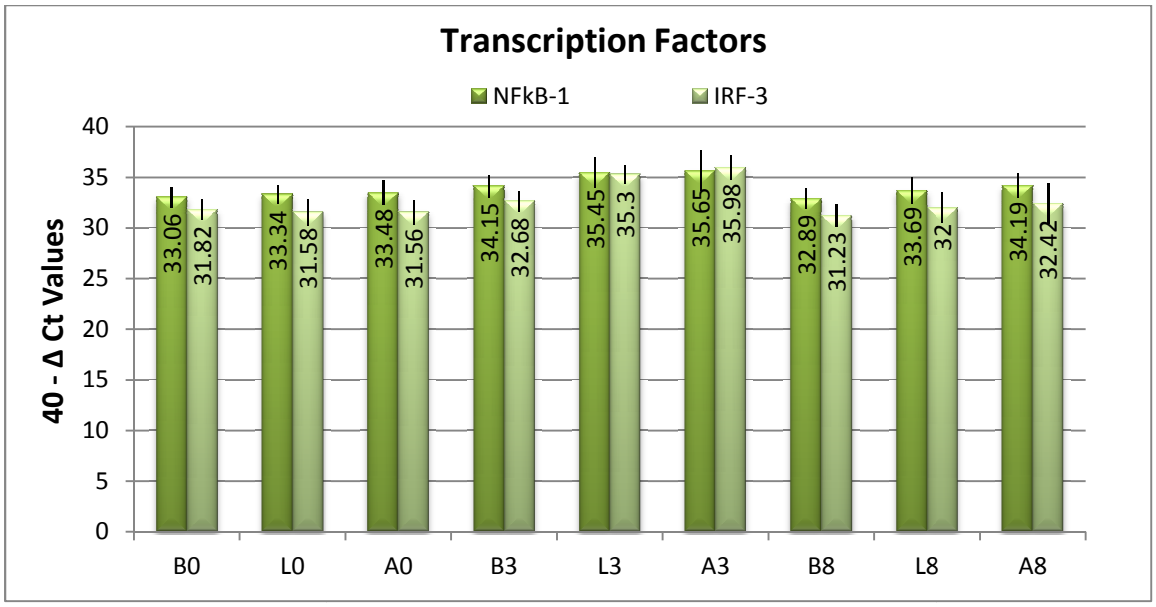

Figure 4.22: 40- ΔCt values of transcription factors NFkβ and IRF-3 in broiler, layer and Aseel without LPS stimulation. β-actin was used as endogenous control.

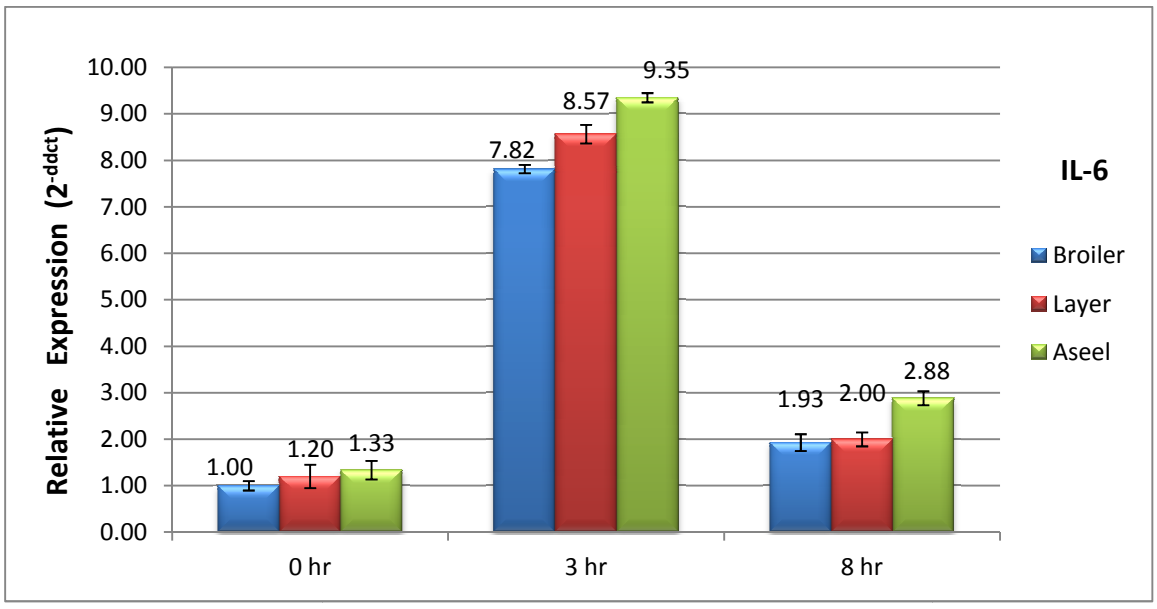

Figure 4.23: Relative Expression levels/ Fold change ($2^{-\Delta\Delta Ct}$) of IL-6, in broiler, layer and Aseel at 0hrs, 3 hrs and 8 hrs of LPS stimulation. β-actin was used as endogenous control.

This observation is in agreement with the expression pattern of most AvBD and TLR genes. These results of the study are in concurrence with similar studies where *in vitro* LPS induction of genes resulted in an increase of NFkB and IRF-3 activity (Tsutsumi and Nagaoka 2002 and Froy 2005). The ligand binding on various membrane TLRs induced transcription factors, activated them and translocated into the nucleus to regulate the expression of immune related genes. The transcription factors that bind to LPS response elements include, NFkB, AP-1, IRF, and STAT (Guha and Mackman 2001). While studying microarray analysis of the expression level of NFkB Bliss *et al* (2005) proved increase in the expression of NFkB in macrophages at 2 hrs after the stimulation of LPS, but also concluded that the transcription factors and the end products of signaling pathway generally increased their expressions with the stimulation, whereas the expression levels of signaling molecules and cellular receptors may not increase or decrease in the same proportion or even may not increase/decrease at all.

Studies conducted by Diamond *et al* (2000) also support the hypothesis that the predicted transcription-factor, NFkB and IRF-3 were associated with induced gene expression by LPS stimulation, as they demonstrated that transcription of the bovine beta-defensin TAP gene is cooperatively regulated by NFkB in response to LPS. Therefore, activating NFkB was probably the signal transduction pathway that induced defensin expression in white blood cells after LPS stimulation. In un-stimulated cells, IκB, an inhibitor of κB, masks the nuclear localization signal on NFkB and thus blocks its nuclear translocation. Upon stimulation, IκB is rapidly phosphorylated and NFkB can translocate to the nucleus, where it turns on the expression of the target gene (Medzhitov 2003).

4.10: Differential gene expression IL6

With the stimulation of LPS, the expression level of interleukin 6 (IL-6) was significantly increased ($p < 0.05$) at 3-hour, and later decreased at 8-hour time point, but it was still higher expressed at 8-hour than that of un-stimulated birds (0-hour time point) (Figure. 4.23). The expression of IL-6 increased to 7.82 to 9.35-fold, at 3- hour after LPS stimulation. The critical regulative inflammatory cytokine, IL-6, was detected to be differentially expressed in broiler and layer at all three time points (0-, 3-, and 8-hrs), with lower expression level in broiler than in layer and Aseel. Increased in concentration of IL-6 in heterophil in LPS-injected birds was also repoted by many workers (Xie *et al* 2000, Leshchinsky and Klasing 2001 and Kogut *et al* 2003 and 2005). The numerous experiments, especially in rat or mouse, illustrated the inductive mechanisms by IL-6 with the stimulation of endotoxins to prove that IL-6 was the major pro-inflammatory cytokine involved (Haziot *et al* 1998, Lyoumi *et al* 1998, Ostberg *et al* 2000, Amrani 1990 and Samad *et al* 1993).

4.11: General remarks

It is well known that the innate immune system not only induces immediate active defense responses but also plays important roles in initiating and instructing the adaptive immune response (Janeway and Medzhitov 2002). Innate immunity limit infections to a minimum in the early stage, by its immediate defensive effect not only make it important, but also critical to the outcome: recovery from infections and restriction from the spread of foreign pathogens.

Tremendous improvements in economic traits have been achieved in commercial

poultry through intensive genetic selection. Unfortunately, selection for rapid growth in chickens has also resulted in higher susceptibility of such stocks to various infectious diseases, as well as ascites and skeletal problems (Julian 1998). Chickens selected for rapid growth show significantly higher morbidity and mortality to various bacterial and viral disease challenges than those with slow growth (Siegel *et al* 1987, Payne *et al* 1992, Rao *et al* 1999, Okuda *et al* 2001 and Songserm *et al* 2003). Broilers have undergone intensive growth selection in the last century and have shown a compromised immune competence (Praharaj *et al* 1996 and Yunis *et al* 2000). Experimental data also indicate that broilers generally show poorer innate (Leshchinsky and Klasing 2001) and adaptive immune responses than the layer chicken(Toro *et al* 1996 and Koenen *et al* 2002). The association of rapid growth with disease susceptibility has also been observed in turkeys (Nestor *et al* 1999). The indigenous breeds of chickens have not undergone intensive selection either for growth or for egg production traits. , and are therefore considered to be more resistant to a variety of diseases than their commercial counterparts. Experiments on the Aseel poultry breed, which is in the most important and renowned indigenous breeds of India, concluded its immunological superiority based on the expression profile of chTLRs in various tissues of chicken (Dhinkar *et al* 2009).

Results of the present study showed significant differences between the strains for expression of some the genes. The layer strain showed higher expression level and stronger activation of chTLRs and AvBDSs and important transcription factors (NFkB-1 and IRF-3) in heterophils with the stimulation of LPS than the broiler strain. Aseel had higher gene expression and stronger activation of chTLRs and AvBDSs in heterophils on LPS stimulation as compared layer strain. Lower gene expression of immune effectors

means lower immune competence.

Modern high growing broiler chicken generally exhibit higher susceptibility to diseases than the commercial laying strains (like White Leghorn strains) which are much smaller in size .Similarly indigenous breeds of chicken because of their low production potential experience higher viability. The differential expression of innate immunity genes as observed in the present study may partially explain the underlying differences in the immune capability of broiler, layer and Aseel, stocks. However further research using wider gene pool may help in further elucidation of the molecular mechanism involved in the full etiology of these differences.

CHAPTER V

Summary

The present investigation was conducted on three divergent strains/stock of chicken viz; improved broiler and layer chicken, and an indigenous stocks of chicken, the Aseel for understanding the immuno-genetic basis of the variable disease resistance/susceptibility observed in such divergent stocks. Differential expression of innate immunity related, toll-like receptor and β- defensin genes were analyzed for constitutive and LPS induced expression using quantitative real-time PCR., LPS, an essential components of the cell wall of all Gram negative bacteria, was used as an Immune indicators against multiple microorganisms to evaluate the differences between the strains for mounting the most effective immune response.

The study was conducted on the birds maintained at poultry breeding farm, GADVASU, Ludhiana. Apparently healthy, six, adult birds each from broiler, layer and Aseel were randomly selected for peripheral blood collection. Birds were from same age group and were under same management and nutritional regimen at the time of sampling, were selected for the study. About 1-2 ml of blood was aseptically, collected from each bird in 15 ml sterile tubes containing EDTA (EDTA, 1.5 mg/ml of blood).

Avian heterophils were isolated from the peripheral blood of chickens (Kogut *et al* 2005) using Ficoll-Hypaque 1.077/1.119 gradient and resuspended in fresh RPMI 1640.

The heterophil cultures were stimulated with 20 µg of LPS per ml of the culture volume and incubated for 0 hrs to study the constitutive relative expression of β-defensin

genes and at 3 hrs and 8 hrs of incubation to study the relative expression on stimulation by LPS. The total RNA was isolated from heterophils at 0hrs, 3hrs and 8hrs of culture incubation using RNeasy mini kit (Qiagen) as per the instructed protocol.

The concentration of recovered RNA was measured using spectrophotometer, nanodrop (Nanodrop 1000, Thermo Scientific). The total RNA with ratio of optical density at 260 nm to that at 280 nm more than 2.0 was accepted for further study. Total RNA concentration was adjusted to 100 ng/ µl using RNase free water. Equal amounts of total RNA from six birds of same strain of chicken were pooled. Further the pooled RNA samples were treated with DNase (Invitrogen, Carlsdad, CA, USA) according to the protocol indicated. Pooled total RNA was used for cDNA synthesis, using First strand synthesis kit (Fermentas, Thermo scientific). The conversion to cDNA was checked by PCR with GAPDH primers. The amplification of 496 bp GAPDH gene fragment from the cDNA indicated the presence of cDNA.

Gene specific primers were designed from the respective gene sequences using online Primer3 software (http://frodo.wi. mit.edu/primer3/), such that the amplicons were about 50 to 150 base pairs in length and the annealing temperature of the reaction could be kept at 60 ° C. β-actin primers were designed for internal control. These primers were custom synthesized by IDT (India).

Differential gene expression for AvBD genes, chTLR genes, transcription factors and pro-inflammatory cytokine IL6, was studied among broiler, layer and Aseel. Experiment was performed in triplicate using an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) using SYBR Green chemistry, with ROX as a passive reference dye.

To estimate the efficiency of PCR reaction, 10-fold serial dilutions was done, starting with 10^7 template copies and ending with 10 copies. PCR efficiency for all the primer sets lied between 94 and 100 %. The heterophil cultures stimulated with 10µg, 20µg, 30µg, 40µg and 50µg of LPS per ml of the culture volume and incubated for 3 hrs were used to study the relative expression of chTLR4 gene and was found to differ in dose - dependent manner. The relative expression was highest at 20 µg/ml dose of LPS, followed by 10µg/ml and 30µg/ml LPS dose, but the expression drastically decreased on further increasing the dose.

The expression of chTLR4 was highly up-regulated expression in all bird strains at 3-hour time point (4.469 to 5.493 folds). The expression decreased to (2.352 to 2.575 folds) at 8-hrs time point, but was still more when compared to its initial expression at 0 hrs, illustrating its function well beyond 8 hrs period. Constitutive expressed of chTLR4 was highest compared to other chTLRs, followed by chTLR5, 2, 3, 7, 15, 21 and 1 in order. The constitutive expression of other chTLR 1, 3, 4, 7 and 15 showed significantly ($p< 0.05$) higher expression in layer compared to broiler. Whereas, chTLR 1 and 7 had significantly higher expression in Aseel compared to layer.

The expression analysis revealed that all the 14 AvBD genes expression were detectable in heterophils and all the 14 AvBD genes and were induced in response to LPS at an early phase i.e. within 0-3hrs period, however the up-regulation in AvBD-9,11,12,13 and 14 was not significant. At 8-hrs after LPS stimulation, the expressions of most AvBDs were down-regulated.

Before stimulation by LPS, the constitutive/ un-induced gene expression of

AvBD-8, 11, 12,13 and 14 showed low level of gene expression in the blood out of which AvBD- 8 was maximally induced by LPS (up to 9.22 to 16.02 fold increase) in 3hrs period and maximally down-regulated to (1.95 to 2.88 folds). Whereas, other early phase low expressing genes AvBD-11, 12, 13, and 14, which did not show sharp up-regulation within 3hrs period of LPS induction continued up-regulated gene expression in late phase i.e. even after 3 hrs period. AvBD-1, 2 and 4 gene expression was among the highest in blood and the up-regulation on induction was only 1.68 folds to 2.88 folds in the early phase.

AvBD-12 showed lower expression (0.78 to 0.81 folds) and AvBD-13 was not induced, with the challenge of LPS at early phase. At the later phase (from 3- to 8-hrs), most AvBDs showed reduced expression, probably to counteract the effects of increased transcription at the early phase, but the overall effect (from 0- to 8-hrs) of LPS stimulation was still regarded as induction for most AvBDs, except for AvBD 3, 4 and 7.

Constitutive expression of AvBD genes in broiler and layer and Aseel reveal that the expression of almost all the genes was higher in layer as compared broiler, and in Aseel when compared to layer. Almost all the AvBDs expression was higher in layer except AvBD-11, 13, 14. However, the difference is significantly higher ($P<0.05$) in layer compared to broiler in case of AvBD-2, 4, 7, 9 and 10 only. Further the constitutive expression of AvBD-6, 7, 8, 9 and 11 in Aseel was significantly higher than layer, with an exception that the expression of AvBD10 was significantly lower. When broiler and Aseel were compared most of the AvBDs expressed at a significantly higher levels in Aseel, except AvBD-5, 11, 12, 13 and 14.

The transcription factors NFkB-1 and IRF-3 were induced to express at early phase (3hrs time point) and expression decreased at the later phase (8hrs time point), which was in agreement with the expression pattern of most AvBD and TLR genes. The expression of IL-6 increases to 7.81 to 9.35-fold, at 3- hrs after LPS stimulation. The critical regulative inflammatory cytokine IL-6 was detected to be differentially expressed in broiler and layer at all three time points (0-, 3-, and 8-hrs), with lower expression level in broiler than in layer and Aseel.

The conclusions which emerged from the study are:

- The expression of the 14 AvBDs, chTLR 1, 2, 3, 4, 5, 7, 15 and 21 was detectable in heterophils using real- time PCR, SYBR Green chemistry.
- Lipopolysaccharide (LPS) of *Salmonella typhimurium* was found to be an *effective* activator of most AvBD genes, *in vitro*, in heterophils.
- Layer strain showed higher expression level and stronger activation of chTLR4, most AvBDs and transcription factors (NFkB-1 and IRF-3) in heterophils with the stimulation of LPS, when compared to broiler.
- Higher expression level and stronger activation of chTLR4, most AvBDs, NFkB-1 and IRF-3, in heterophils was observed, with the stimulation of LPS in Aseel compared to both layer and broiler.

Therefore Aseel is immuno-genetically more disease resistant compared to layer and broiler. Layer is immuno-genetically more disease resistant compared broiler.

References

Abasht B, Sandford E and Arango J. 2009. Extent and consistency of linkage disequilibrium and identification of DNA markers for production and egg quality traits in commercial layer chicken populations. *BMC Genomics* **10**: 314-23.

Abbas A K and Lichtman A H. 2003. Cellular and molecular immunology. 5th edn Saunders, Philadelphia.

Ajuwon K, Banz W and Winters T. 2009. Stimulation with Peptidoglycan induces interleukin 6 and TLR2 expression and a concomitant down regulation of expression of adiponectin receptors 1 and 2 in 3T3-L1 adipocytes. *Journal of Inflammation* **6**:1-12.

Akira S, Takeda K and Kaisho T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nature Immunology* **2**: 675-80.

Akira S and Takeda K. 2004. Toll-like receptor signaling. *Nature Reviews Immunology* **4**: 499-511.

Amrani D L. 1990. Regulation of fibrinogen biosynthesis: glucocorticoid and interleukin-6 control. *Blood Coagulation and Fibrinolysis* **1**: 443-46.

Anderson K V, Bokla L and Nusslein C. 1985. Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. *Cell* **42**: 791-8.

Baetz A, Frey M, Heeg K and Dalpke A H. 2004. Suppressor of cytokine signaling. SOCS. proteins indirectly regulate toll-like receptor signaling in innate immune cells. *Journal of Biological Chemistry* **279**: 54708-15.

Bals R, Wang X, Wu Z, Freeman T, Bafna V, Zasloff M and Wilson J M. 1998. Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *Journal of Clinical Investigation* **102**: 874-80.

Banchereau J and Steinman R. 1998. Dendritic cells and the control of immunity. *Nature* **392:** 245–52.

Bayyari G R, Huff W E, Rath N C, Balog J M, Newberry L A , Villines J D and Skeeles J K. 1997. Immune and physiological responses of turkeys with green-liver osteomyelitis complex. *Poultry Science* **76**: 280–88.

Berczi I. 1998. Neurohormonal host defense in endotoxin shock. *Annals of the New York Acadamy of* Science **840**: 787–802.

Biragyn A, Surenhu M, Yang D, Ruffini P A, Haines B A, Klyushnenkova E, Oppenheim JJ and Kwak LW. 2001. Mediators of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens. *Journal of Immunology* **167**: 6644-53.

Bliss T W, Dohms J E, Emara M G and Keeler C L. 2005. Gene expression profiling of avian macrophage activation. *Veterinary Immunology and Immunopathology* **105**: 289-99.

Boman H G. 2003. Antibacterial peptides: basic facts and emerging concepts. *Journal of Internal Medicine* **254**: 197-215.

Boyd Y, Goodchild M, Morroll S and Bumstead N. 2001. Mapping of the chicken and mouse genes for tolllike receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. *Immunogenetics.* **52:** 294–98.

Brandt E, Woerly G, Younes A B, Loiseau S and Capron M. 2000. IL-4 production by human polymorphonuclear neutrophils. *Journal of Leukocyte Biology* **68**: 125–30.

Brogden K A. 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology* **3**: 238–50.

Brown K L and Hancock R E. 2006. Cationic host defense. antimicrobial. peptides. *Current Opinion in Immunology* **18**: 24-30.

Bulyk M L. 2003. Computational Prediction of Transcription Factor Binding Site Locations. *Genome Biology* **5**: 201.1-201.11

Bustin S A. 2002. Quantification of mRNA using real-time reverse transcription PCR. RT-PCR.: trends and problems. *Journal of Molecular Endocrinology **29***:23-39.

Chen H, Xu Z, Peng L, Fang X, Yin X, Xu N and Cen P. 2006. Recent advances in the research and development of human defensins. *Peptides* **27**: 931-40.

Cobrin G M and Abreu M T. 2005. Defects in mucosal immunity leading to Crohn's disease. *Immunology Reviews* **206**: 277-95.

Cowland J B and Borregaard N. 1999. The individual regulation of granule protein mRNA levels during neutrophil maturation explains the heterogeneity of neutrophil granules. *Journal of Leukocyte Biology* **66**: 989-95.

Derache C, Esnault E, Bonsergent C, Le V Y, Quere P and Lalmanach A C. 2009. Differential modulation of beta-defensin gene expression by Salmonella Enteritidis in intestinal epithelial cells from resistant and susceptible chicken inbred lines. *Developmental and Comparative Immunology* **33:** 959- 66.

Dhinakar R G, Chozhavel T M, Kumaran K and Elankumaran S. 2009. Expression profile of Toll- Like Receptor mRNA in an indigenous Aseel of chicken in India. *International Journal of Poultry Science*.**8**: 651-55.

Diamond G, Russell J P and Bevins C L. 1996. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proceedings of the National Academy of Sciences USA* **93**: 5156-60.

Diamond G, Zasloff M, Eck H, Brasseur M, Maloy W L and Bevins CL. 1991. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proceedings of the National Academy of Sciences USA* **88**: 3952-56.

Diamond G, Kaiser V, Rhodes J, Russell J P and Bevins C L. 2000. Transcriptional Regulation of β-Defensin Gene Expression in Tracheal Epithelial Cells. *Infection and Immunity* **68**: 113-19.

Diekema D J, Brueggemann A B and Doern G V. 2000. Antimicrobial-drug use and changes in resistance in Streptococcus pneumoniae. *Emerging Infectious Diseases* **6**: 552-56.

Dillon S, Agrawal A, Van Dyke T, Landreth G, McCauley L, Koh A, Maliszewski C, Akira S and Pulendran B. 2004. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *Journal of Immunology* **172:** 4733-35.

Drakesmith H, Chain B and Beverley P. 2000. How can dendritic cells cause autoimmune disease? *Immunology Today* **21**: 214-17.

Dunne A and O'Neill L A. 2005. Adaptor usage and Toll-like receptor signaling specificity. *FEBS Letters* **579**: 3330-35.

Durell S R, Raghunathan G and Guy H R. 1992. Modeling the ion channel structure of cecropin. *Biophysics Journal* **63**: 1623-31.

Durr M and Peschel A. 2002. Chemokines meet defensins: the merging concepts of chemoattractants and antimicrobial peptides in host defense. *Infection and Immunity* **70:** 6515–17.

Ebers K L, Zhang C Y, Zhang M Z, Bailey R H and Zhang S. 2009. Transcriptional profiling avian beta-defensins in chicken oviduct epithelial cells before and after infection with Salmonella enterica serovar Enteritidis. *BMC Microbiology.* **9**:153.

Eisenhauer P, Harwig S S, Szklarek D, Ganz T and Lehrer RI. 1990. Polymorphic

expression of defensins in neutrophils from outbred rats. *Infection and Immunity* **58**: 3899-902.

Eisenhauer P B, Harwig S S, Szklarek D, Ganz T, Selsted M E and Lehrer R I. 1989. Purification and antimicrobial properties of three defensins from rat neutrophils. *Infection and Immunity* **57**: 2021-27.

Eisenhauer P B and Lehrer R I. 1992. Mouse neutrophils lack defensins. *Infection and Immunity* **60**: 3446-7.

Evans E W, Beach F G, Moore K M, Jackwood M W, Glisson J R and Harmon B G. 1995. Antimicrobial activity of chicken and turkey heterophil peptides CHP1, CHP2, THP1, and THP3. *Veterinary Microbiology* **47**: 295-303.

Faure E, Equils O and Sieling P. 2000. Bacterial lipopolysaccharide activates NF-kB through Toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *Journal of Biological Chemistry* **275:** 11058 - 63.

Ficken M D and Barnes H J. 1989. Acute airsacculitis in turkeys inoculated with Pasteurella multocida. Veterinary Pathology **26**: 231–237.

Freeman W M, Walker S J and Vrana K E. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* **26**: 112-22.

Froy O. 2005. Regulation of Mammalian Defensin Expression by Toll-like Receptor-Dependent and Independent Signaling Pathways. *Cellular Microbiology* **7**: 1387-1397.

Fukui A, Inoue N, Matsumoto M, Nomura M, Yamada K, Matsuda Y, Toyoshima K and Seya T. 2001. Molecular cloning and functional characterization of chicken toll-like receptors. A single chicken toll covers multiple molecular patterns. *Journal of Biological Chemistry* **276**: 47143-49.

Gangloff M and Gay N J. 2004. MD-2: The Toll 'Gatekeeper' in Endotoxin Signaling. *Trends in Biochemical Sciences* **29:** 294-300.

Ganz T, Selsted ME and Lehrer RI. 1990. Defensins. *European Journal of Haematology* **44**: 1-8.

Ganz T. 2002. Immunology. Versatile defensins. *Science* **298**: 977-9.

Ganz T. 2003. Defensins: antimicrobial peptides of innate immunity. *Nature Reviews Immunology* **3**: 710–20.

Ganz T. 2004. Defensins: antimicrobial peptides of vertebrates. *C R Biology* **327**: 539-49.

Ganz T. 2005. Defensins and other antimicrobial peptides: a historical perspective and an update. *Comb Chem High Throughput Screen* **8:** 209-17.

Garcia J R, Krause A, Schulz S, Rodriguez F J, Kluver E, Adermann K, Forssmann U, Frimpong A, Bals R and Forssmann WG. 2001. Human beta- defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB Journal* **15**: 1819-21.

Gay N and Keith F. 1991. Drosophila *Toll* and IL-1 receptor. *Nature* **351:** 355–56.

Giuliani A, Pirri G and Nicoletto S F. 2007. Antimicrobial peptides: An overview of a promising class of therapeutics. *European Journal of Biology* **2**: 1–33.

Gordon Y J, Romanowski E G and McDermott A M. 2005. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Current Eye Research* **30**: 505–515.

Graaf A, Netea M, Verschueren I, Jos W and Kullberg B. 2005. Differential cytokine production and Toll-like receptor signaling pathways by *Candida albicans* bloostoconidia and hyphae. *Infection and Immunity* **73:** 7458-64.

Groisman E A, Parra-Lopez C, Salcedo M, Lipps C J and Heffron F. 1992. Resistance to host antimicrobial peptides is necessary. *Proceedings of the National Academy of Sciences USA* **89**: 11939–43.

Guha M and Mackman N. 2001. LPS Induction of Gene Expression in Human Monocytes. *Cellular Signalling* **13:** 85-94.

Gunn J S. 2008. The Salmonella Pmr AB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends in Microbiology* **16**: 284–290.

Hackett C J. 2003. Innate immune activation as a broad-spectrum biodefense strategy: prospects and research challenges. *Journal of Allergy and Clinical Immunology* **112**: 686-94.

Hale J D and Hancock R E. 2007. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Review of Anti-infective Therapy* **5**: 951–959.

Hancock R E and Sahl H G. 2006. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology* **24**: 1551–1557.

Hancock R E and Lehrer R. 1998. Cationic peptides: a new source of antibiotics. *Trends in Biotechnology* **16**: 82–88.

Hancock R E and Rozek A. 2002. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiology Letters* **206:** 143–9.

Hancock R E W and Scott M G. 2000. The Role of Antimicrobial Peptides in Animal Defenses. *Proceedings of the National Academy of Sciences USA* 97: 8856-8861

Hancock R E and Diamond G. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends in Microbiology* **8:** 402–410.

Hao H N, Zhao J, Lotoczky G, Grever W E and Lyman W D. 2001. Induction of human beta- defensin-2 expression in human astrocytes by lipopolysaccharide and cytokines. *Journal of Neurochemistry* **77**: 1027-35.

Harder J, Bartels J, Christophers E and Schroder JM. 2001. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *Journal of Biological Chemistry* **276**: 5707-13.

Harder J, Meyer-Hoffert U, Teran LM, Schwichtenberg L, Bartels J, Maune S and Schroder JM. 2000. Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *American Journal of Respiratory Cell Molecular Biology* **22**: 714-21.

Harwig S S, Swiderek K M, Kokryakov V N, Tan L, Lee T D, Panyutich EA, Aleshina G M, Shamova O V and Lehrer R I. 1994. Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. *FEBS Letters* **342**: 281-5.

Harwig S S, Kokryakov V N, Swiderek K M, Aleshina G M, Zhao C and Lehrer R I. 1995. Prophenin-1, an exceptionally proline-rich antimicrobial peptide from porcine leukocytes. *FEBS Letters* **362**: 65-69.

Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, Guiet C, Briere F, Vlach J, Lebecque S, Trinchieri G and Bates EE. 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *Journal of Immunology* **174:**2942-50.

Hashimoto C, Hudson K L and Anderson K V. 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52:** 269-79.

Haziot A, Lin X Y, Zhang F and Goyert S M. 1998. The induction of acute phase proteins by lipopolysaccharide uses a novel pathway that is CD14-independent. *Journal of Immunology* **160**: 2570-2.

He H, Genovese K J, Nisbet D J and Kogut M H. 2006. Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes. *Molecular Immunology* **43**: 783-9.

Higgs R, Cormican P, Cahalane S, Allan B, Lloyd A T, Meade K, James T, Lynn D J, Babiuk L A and O'farrelly C. 2006. Induction of a novel chicken Toll-like receptor following Salmonella enterica serovar Typhimurium infection. *Infection and Immunity* **74**: 1692-8.

Higgs R, Lynn D J, Gaines S, McMahon J, Tierney J, James T, Lloyd A T, Mulcahy G and O'Farrelly C. 2005. The synthetic form of a novel chicken beta-defensin identified in silico is predominantly active against intestinal pathogens. *Immunogenetics* **57**: 90-8.

Hirschfeld M, Weis J, Toshchakov V, Salkowski C, Cody M, Ward D, Qureshi N, Michalek M and Vogel N. 2001. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infection and Immunity* **69:** 1477-79.

Huang H J and Matsumoto M. 2000. Nonspecific innate immunity against Escherichia coli infection in chickens induced by vaccine strains of Newcastle disease virus. *Avian Diseases* **44**: 790-96.

Huang L, Proske R J and McDermott A M. 2004. Functional roles of the epithelial-derived antimicrobial- derived peptide LL-37 at the ocular surface. Investigative Ophthalmology and Visual Science **45**: 45-55

Iqbal M, Philbin V J and Smith A L. 2005. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Veterinary Immunology and Immunopathology* **104**: 117-27.

Janeway CA Jr and Medzhitov R. 2002. Innate immune recognition. *Annual Review of Immunology* **20**: 197-216.

Janssens S and Beyaert R. 2003. Functional diversity and regulation of different interleukin- 1 receptor-associated kinase. IRAK. family members. *Molecular Cell* **11**: 293-302.

Jones D E and Bevins C L. 1992. Paneth cells of the human small intestine express an antimicrobial peptide gene. *Journal of Biological Chemistry* **267**: 23216-25.

Julian R J. 1998. Rapid growth problems: ascites and skeletal deformities in broiler. *Poultry Science* **77**: 1773-80.

Kaiser M G, Cheeseman J H, Kaiser P and Lamont S J. 2006. Cytokine expression in chicken peripheral blood mononuclear cells after in vitro exposure to Salmonella enterica serovar Enteritidis. *Poultry Science* **85:** 1907-11.

Kaiser P. 2007. The avian immune genome-a glass half-full or halfempty? *Immunology* **117**: 221–230.

Kaiser V and Diamond G. 2000. Expression of Mammalian Defensin Genes. *Journal of Leukocyte Biology* **68:** 779-84.

Katial R, Sachanandani D, Pinney C and Lieberman M. 1998. Cytokine production in cell culture by peripheral blood mononuclear cells from immunocompetent hosts. *Clinical and diagnostic laboratory immunology* **5:** 78-81.

Kaufmann H, Medzhitov R and Gordon S. (2004. The Innate Immune Response to Infection. pp 456. ASM Press. Washington DC.

Kawai T and Akira S. 2006. TLR signaling. *Cell Death Differ* **13**: 816-25.

Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt P, Sato S, Hoshino K and Akira S. 2001. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *Journal of Immunology* **167:** 5887.

Keestra A M, Zoete M R, Aubel R A and Putten J P. 2007. The central leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. *Journal of Immunology* **178:** 7110–19.

Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, Yoshida H, Kubo M and Yoshimura A. 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* **17**: 583-91.

Kirschning C J, Wesche H, Merrill A T and Rothe M. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *The Journal of Experimental Medicine* **188**: 2091-97.

Klinman D M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nature Reviews Immunology* **4**: 249-58.

Kobayashi K, Hernandez L D, Galan J E, Janeway C A Jr, Medzhitov R and Flavell R A. 2002. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* **110**:191-202.

Koenen M E, Boonstra-Blom A G and Jeurissen S H. 2002. Immunological differences between layer- and broiler-type chickens. *Veterinary Immunology and*

Immunopathology **89**: 47-56.

Kogut M H, Lowry V K, Moyes R B, Bowden L L, Bowden R, Genovese K and Deloach J R. 1998. Lymphokine augmented activation of avian heterophils. *Poultry Science* **77:** 964–971.

Kogut M H, Rothwell L and Kaiser P. 2003. Differential regulation of cytokine gene expression by avian heterophils during receptor mediated phagocytosis of opsonized and nonopsonized Salmonella enteritidis. *Journal of Interferon Cytokine Research* **23:** 319–27.

Kogut M H, Iqbal M, He H, Philbin V, Kaiser P and Smith A. 2005. Expression and function of Toll-like receptors in chicken heterophils. *Developmental and Comparative Immunology* **29**: 791-807.

Kraus D and Peschel A. 2008. Staphylococcus aureus evasion of innate antimicrobial defense. *Future Microbiology* **3**: 437– 51.

Laverty G, Gorman S P and Gilmore F. 2011. The Potential of Antimicrobial Peptides as Biocides. *International Journal of Molecular Science* **12:** 6566-96.

Lehrer R I and Ganz T. 2002. Defensins of vertebrate animals. *Current Opinion in Immunology* **14**: 96-98.

Lehrer R I. 2004. Primate defensins. *Nature Reviews Microbiology* **2:** 727-38.

Lemaitre B, Nicolas E, Michaut L, Reichhart J and Hoffmann J. 1996. The dorsoventral regulatory gene cassette Spaetzle/Toll/Cactus controls the potent antifungal response in Drosophila adults. *Cell* **86:** 973–83.

Leng Q and Mixson A J. 2005. Modified branched peptides with a histidine-rich tail enhance in vitro gene transfection. Nucleic Acids Res **33**:40-42

Leshchinsky T V and Klasing K C. 2001. Divergence of the inflammatory response in two types of chickens. *Developmental and Comparative Immunology* **25**: 629-38.

Leveque G, Forgetta V, Morroll S, Smith A L, Bumstead N, Barrow P, Loredo-Osti J C, Morgan K and Malo D. 2003. Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica serovar Typhimurium infection in chickens. *Infection and Immunity* **71**: 1116-24.

Lin Y, Lee H, Berg A, Lisanti M, Shapiro L and Scherer P. 2000. The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *Journal of Biological Chemistry* **275:** 24255-63.

Linzmeier R, Ho C H, Hoang B V and Ganz T. 1999. A 450-kb contig of defensin genes on human chromosome 8p23. *Gene* **233:** 205-11.

Liu L, Roberts A A and Ganz T. 2003. By IL-1 signaling, monocyte-derived cells dramatically enhance the epidermal antimicrobial response to lipopolysaccharide. *Journal of Immunology* **170:** 575-80.

Liu L, Zhao C, Heng H H Q and Ganz T. 1997. The Human β-Defensin-1 and α-Defensins are Encoded by Adjacent Genes: Two Peptide Families with Differing Disulfide Topology Share a Common Ancestry. *Genomics* **43:** 316-320.

Lorenzoni A G, and Wideman R F. 2008. Intratracheal administration of bacterial lipopolysaccharide elicits pulmonary hypertension in broilers with primed airways. *Poultry Science* **87:** 645–654.

Lynn D J, Lloyd A T and O'Farrelly C. 2003. In silico identification of components of the Toll-like receptor. TLR. signaling pathway in clustered chicken expressed sequence tags. ESTs. *Veterinary Immunology and Immunopathology* **93:** 177-84.

Lynn D J, Higgs R, Gaines S, Tierney J, James T, Lloyd A T, Fares M A, Mulcahy G and O'Farrelly C. 2004. Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. *Immunogenetics* **56:** 170-77.

Lynn D J, Higgs R, Lloyd A T, O'Farrelly C, Hervé-Grépinet V, Nys Y, Brinkman F S, Yu P L, Soulier A, Kaiser P, Zhang G, and Lehrer R I. 2007. Avian beta-defensin nomenclature: A community proposed update. *Immunology Letters* **110:** 86-89.

Lyoumi S, Tamion F, Petit J, Dechelotte P, Dauguet C, Scotte M, Hiron M, Leplingard A, Salier JP, Daveau M and Lebreton J P. 1998. Induction and modulation of acute-phase response by protein malnutrition in rats: comparative effect of systemic and localized inflammation on interleukin-6 and acute-phase protein synthesis. *Journal of Nutrition* **128**: 166-74.

Mansell A, Smith R, Doyle S L, Gray P, Fenner J E, Crack P J, Nicholson S E, Hilton D J, O'Neill L A and Hertzog P J. 2006. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nature Immunology* **7**: 148-55.

Matejuk A Q, Leng M D. Begum M C, Woodle P Scaria, Chou ST and Mixson A J. 2010. Peptide-based Antifungal Therapies against Emerging Infections. *Drugs Future* **35**(3): 197.

Mathews M, Jia H P, Guthmiller J M, Losh G, Graham S, Johnson G K, Tack BF and McCray P B Jr. 1999. Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. *Infection and Immunology* **67**: 2740-5.

Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A and Seya T. 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *Journal of Immunology* **171**: 3154.

Matsushita M and Fujita T. 1995. Cleavage of the third component of complement. C3. by mannose-binding protein-associated serine protease. MASP. with subsequent complement activation. *Immunobiology* **194**: 443-48.

Matsuyama K and Natori S. 1988. Purification of three antibacterial proteins from the culture medium of NIH-Sape-4, an embryonic cell line of Sarcophaga peregrina. *Journal of Biological Chemistry* **263**: 17112-16.

Medzhitov R, Preston-Hurlburt P and Janeway C A. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**: 394-97.

Medzhitov R. 2001. Toll-like receptors and innate immunity. *Nature Reviews Immunology* **1**: 135-45.

Medzhitov R. 2003. The Innate Immune System. In: Paul, W. E Fundamental Immunology. 5th edn . pp 497. Lippincott Williams & Wilkins, Philadelphia.

Mendez E, Moreno A, Colilla F, Pelaez F, Limas GG, Mendez R, Soriano F, Salinas M and Haro C. 1990. Primary structure and inhibition of protein synthesis in eukaryotic cell- free system of a novel thionin, gamma-hordothionin, from barley endosperm. *European Journal of Biochemistry* **194**: 533-39.

Montali R J. 1988. Comparative pathology of inflammation in the higher vertebrates (reptiles, birds and mammals). *Journal of Comparative Pathology* **99**: 1–20.

Moody D E. 2001. Genomics Techniques: An Overview of Methods for the Study Gene Expression. *Journal of Animal Science* **78:** 128-135

Musa H, Yunhua X, Baoying D, Xiaoyan K, Chunhong Z and Guoqiang Z. 2009. Cytokine gene expression of peripheral blood lymphocytes stimulated by lipopolysaccharide. *African Journal of Biotechnology* **8:** 995-99.

Muzio M and Mantovani A. 2001. The Toll receptor family. *Allergy* **56**: 103-08.

Nestor K E, Saif Y M, Anderson J W, Patterson R A and Li Z. 1999. Variation in resistance to Pasteurella multocida among turkey lines. *Poultry Science* **78**: 1377-79.

Neuhaus F C and Baddiley J A. 2003. Continuum of anionic charge: Structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiology and Molecular Biology Reviews* **67:** 686–23.

Nguyen T X, Cole A M and Lehrer R I. 2003. Evolution of primate theta-defensins: a serpentine path to a sweet tooth. *Peptides* **24**: 1647-54.

Nikolaus S, Bauditz J, Gionchetti P, Witt C, Lochs H and Schreiber S. 1998. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation. *Gut* **42**: 470–476.

O' Neill L. 2002. Toll loke receptor signal transduction and the tailoring of innate immunity. *Trends in Immunology* **23:** 296-300.

Okuda Y, Ono M, Yazawa S, Imai Y, Shibata I and Sato S. 2001. Pathogenicity of serotype 1 fowl adenovirus in commercial broiler chickens. *Avian Diseases* **45:**819-27.

Oliveira R B, Moraes M O, Oliveira E B, Sarno E N, Nery J A and Sampaio E P. 1999. Neutrophils isolated from leprosy patients release TNF-alpha and exhibit accelerated apoptosis in vitro. *Journal of Leukocyte Biology* **65**: 364–371.

O'Neil D A, Porter E M, Elewaut D, Anderson G M, Eckmann L, Ganz T and Kagnoff M F. 1999. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *Journal of Immunology* **163**: 6718-24.

O'Neill L A, Fitzgerald K A and Bowie A G. 2003. The Toll-IL-1 receptor adaptor family grows to five members. *Trends in Immunology* **24**: 286-90.

Oppenheim J J, Biragyn A, Kwak L W and Yang D. 2003. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. *Annals of the Rheumatic Diseases* **2**: 17-21.

Oshiumi H, Tsujita T, Shida K, Matsumoto M, Ikeo K and Seya T. 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, Fugu rubripes, genome. *Immunogenetics* **54**: 791-800.

Ostberg J R, Taylor S L, Baumann H and Repasky EA. 2000. Regulatory effects of fever-range whole-body hyperthermia on the LPS-induced acute inflammatory response. *Journal of Leukocyte Biology* **68**: 815-20.

Overbergh L, Giulietti A, Valckx D, Decallonne R, Bouillon R and Mathieu C. 2003. The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *Journal of Biomolecular Techniques* **14**: 33-43.

Parra C, Baer M and Groisman E. 1993. Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in Salmonella typhimurium. *EMBO J.* **12:**4053–62.

Pasare C and Medzhitov R. 2004. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infection* **6:**1382-87.

Payne LN, Gillespie A M and Howes K. 1992. Myeloid leukaemogenicity and transmission of the HPRS-103 strain of avian leukosis virus. *Leukemia* **6:**1167-76.

Philbin V J, Iqbal M, Boyd Y, Goodchild MJ, Beal R K, Bumstead N, Young J and Smith A L. 2005. Identification and characterization of a functional, alternatively spliced toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* **114:**507–521.

Ponchel F, Toomes C, Bransfield K, Leong F T, Douglas S H, Field S L, Bell S M, Combaret V, Puisieux A, Mighell A J, Robinson P A, Inglehearn CF, Isaacs J D and Markham A F. 2003. Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. *BMC Biotechnology* **3:** 18-21.

Praharaj N K, Gross W B, Dunnington E A, Nir I and Siegel P B. 1996. Immunoresponsiveness of fast-growing chickens as influenced by feeding regimen. *British Poultry Science* **37:**779-86.

Pulendran B, Kumar P, Cutler C, Mohamadzadeh M, Van Dyke T and Banchereau J. 2001. Lipopolysaccharides from distinct pathogens induce different classes of immune responses *in vivo*. *Journal of Immunology* **167:** 5067–76.

Radhakrishnan Y, Hamil KG, Yenugu S, Young SL, French FS, Hall SH. 2005. Identification, characterization, and evolution of a primate beta-defensin gene cluster. *Genes and Immunity* **6:**203-10.

Raj P A and Dentino A R. 2002. Current status of defensins and their role in innate and adaptive immunity. *FEMS Microbiology Letters* **206:**9-18.

Ramasamy K T, Reddy M R, Raveendranathan D N, Murugesan S, Chatterjee R N, Ullengala R and Haunshi S. 2010. Differential expression of Toll-like receptor mRNA in White Leghorn and indigenous chicken of India. *Veterinary Research Communication* **34:**633–39.

Ramasamy K T, Verma P and Reddy M R. 2012. Differential gene expression of antimicrobial peptides β defensins in the gastrointestinal tract of Salmonella serovar Pullorum infected broiler chickens. *Veterinary Research Communication* **36:**57-62.

Rao S V, Praharaj N K, Reddy M R and Sridevi B. 1999. Immune competence, resistance to Escherichia coli and growth in male broiler parent chicks fed different levels of crude protein. *Veterinary Research Communication* **23:**323-26.

Rath N C, Huff G R, Balog J M and Huff W E. 1998. Fluorescein isothiocyanate staining and characterization of avian heterophils. *Veterinary Immunology and Immunopathology* **64:**83–95.

Roach J C, Glusman G, Rowen L, Kaur A, Purcell M K, Smith K D, Hood L E and Aderem A. 2005. The evolution of vertebrate Toll-like receptors. *Proceedings of National Academy of Science* **102:**9577-82.

Russell J P, Diamond G, Tarver A P, Scanlin T F and Bevins C L. 1996. Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor alpha. *Infection and Immunity* **64:**1565-68.

Samad F, Bergtrom G, Eissa H and Amrani DL. 1993. Stimulation of chick hepatocyte fibronectin production by fibroblast-conditioned medium is due to interleukin 6. *Biochimica et Biophysica Acta* **1181:**207-13.

Schmid H, Cohen C D, Henger A, Irrgang S, Schlondorff D and Kretzler M. 2003. Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. *Kidney International* **64:**356-60.

Schnare M, Barton G M, Holt A C, Takeda K, Akira S and Medzhitov R. 2001. Toll-like receptors control activation of adaptive immune responses. *Nature Immunology* **2:**947-50.

Schutte B C and McCray P B . 2002. beta-defensins in lung host defense. *Annual Review of Physiology* **64:**709-48.

Schwalbe R A, Dahlback B, Coe JE and Nelsestuen G L. 1992. Pentraxin family of proteins interact specifically with phosphorylcholine and/or phosphorylethanolamine. *Biochemistry* **31:**4907-15.

Scott M G, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, Wang A, Lee K, Doria S, Hamill P, Yu J J, Li Y, Donini O, Guarna MM, Finlay B B, North J R and Hancock R E .2007. An anti-infective peptide that selectively modulates the innate immune response. *Nature Biotechnology* **25:** 465–472.

Selsted M E, Szklarek D and Lehrer R I. 1984. Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. *Infection and Immunity* **45:**150-54.

Selsted M E, Tang Y Q, Morris W L, McGuire P A, Novotny M J, Smith W, Henschen A H and Cullor J S. 1993. Purification, primary structures, and antibacterial activities of beta- defensins, a new family of antimicrobial peptides from bovine neutrophils. *Journal of Biological Chemistry* **268:**6641-48.

Shai Y. 2002. From innate immunity to *de-novo* designed antimicrobial peptides. *Current Pharmceutical Design* **8:** 715–25.

Siegel P B, Katanbaf M N, Anthony N B, Jones D E, Martin A, Gross W B and Dunnington E A. 1987. Responses of chickens to Streptococcus faecalis: genotype-housing interactions. *Avian Dis*eases **31:**804-08.

Singh P K, Jia H P, Wiles K, Hesselberth J, Liu L, Conway B A, Greenberg E P, Valore E V, Welsh M J, Ganz T, Tack B F and McCray P B. 1998. Production of beta-defensins by human airway epithelia. *Proceedings of National Academy of Science* **95:**14961-66.

Songserm T, van Roozelaar D, Kant A, Pol J, Pijpers A and Huurne A. 2003. Enteropathogenicity of Dutch and German avian reoviruses in SPF white leghorn chickens and broilers. *Veterinary Research* **34:**285-95.

Stein D, Roth S, Vogelsang E and Nusslein C. 1991. The polarity of the dorsoventral axis in the Drosophila embryo is defined by an extracellular signal. *Cell* **65:**725-35.

Steiner H, Hultmark D, Engstrom A, Bennich H and Boman HG. 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* **292:**246-48.

Sugiarto H and Yu P. 2004. Avian Antimicrobial Peptides: The Defense Role of β-Defensins. *Biochemical and Biophysical Research Communications* **323:** 721- 27.

Sunwoo H H, Nakano T, Dixon WT and Sim J S. 1996. Immune responses in chickens against lipopolysaccharide of Escherichia coli and Salmonella typhimurium. *Poultry Science* **75:**342–45.

Suzuki T, Higgins PJ and Crawford DR. 2000. Control selection for RNA quantitation. *Biotechniques* **29:**332-37.

Takahashi K, Yodogawa S and Akiba Y. 1995. Effect of dietary protein concentration on response to Escherichia coli endotoxin in broiler chickens. *British Journal of Nutrition* **74:**173–82.

Takeda K and Akira S. 2005. Toll-like receptors in innate immunity. *International*

*Immunol*ogy **17:**1-14

Takeda K, Kaisho T and Akira S. 2003. Toll-like receptors. *Annual Review of Immunology* **21:**335-76.

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K and Akira S. 1999. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram- positive bacterial cell wall components. *Immunity* **11:**443-51.

Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, Takeda K and Akira S. 1999. TLR6: A novel member of an expanding toll-like receptor family. *Gene* **231:**59-65.

Tang Y Q, Yuan J, Osapay G, Osapay K, Tran D, Miller C J, Ouellette A J and Selsted M E. 1999. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* **286:**498-502.

Tapping R I, Akashi S, Miyake K, Godowski P J and Tobias P S. 2000. Toll-like receptor 4, but not toll-like receptor 2, is a signaling receptor for Escherichia and Salmonella lipopolysaccharides. *Journal of Immunology* **165:**5780-87

Tizard I R. 2004. Veterinary Immunology: An Introduction. 7th edn Saunders, Philadelphia

Tomasinsig L and Zanetti M. 2005. The cathelicidins--structure, function and evolution. *Current Protein Peptide Science* **6:**23-34.

Toro H, Reyes E, Redmann T and Kaleta E F. 1996. Local and systemic specific antibody response of different chicken lines after ocular vaccination against infectious bronchitis. *Zentralbl Veterinarmed B* **43:**449-54.

Torres A M, Kuchel P W. 2004. The beta-defensin-fold family of polypeptides. *Toxicon* **44:**581-88.

Toth T E, Siegel P B and Veit H. 1987. Cellular defense of the avian respiratory system. Influx of phagocytes: elicitation versus activation. *Avian Diseases* **31:**861–67.

Tsutsumi Y and Nagaoka I. 2002. NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation. *Journal of Leukocytw Biology* **71:**154-62.

Underhill D M and Ozinsky A. 2002. Toll-like receptors: key mediators of microbe detection. *Current Opinion in Immunology* **14:**103-10.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by

geometric averaging of multiple internal control genes. *Genome Biology* **3:**1-12.

Vankeerberghen A, Nuytten H, Dierickx K, Quirynen M, Cassiman J J and Cuppens H. 2005. Differential induction of human beta-defensin expression by periodontal commensals and pathogens in periodontal pocket epithelial cells. *Journal of Periodontology* **76:**1293- 303.

Wada A, Ogushi K, Kimura T, Hojo H, Mori N, Suzuki S, Kumatori A, Se M, Nakahara Y, Nakamura M, Moss J and Hirayama T. 2001. Helicobacter pylori-mediated transcriptional regulation of the human beta-defensin 2 gene requires NF-kappaB. *Cell Microbiology* **3:**115-23.

Wang D, Westerheide S, Hanson J, and Baldwin Jr. 2000. Tumor necrosis factor- α induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. *Journal of Biological Chemistry* **275:** 32592-97.

Wang J, Jorgensen F, Ellingsen E, Almlof M, Thiemermann C, Foster S, Aasen A and Solberg R. 2001. Peptidoglycan primes for LPS-induced release of pro-inflammatory cytokines in whole human blood. *Shock* **16:**178-82.

Wehkamp K, Schwichtenberg L, Schroder J M and Harder J. 2006. Pseudomonas aeruginosa- and IL-1beta-mediated induction of human beta-defensin-2 in keratinocytes is controlled by NF-kappaB and AP-1. *Journal of Investigative Dermatology* **126:**121-7.

Wesche H, Gao X, Li X, Kirschning C J, Stark G R and Cao Z. 1999. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase. IRAK. family. *Journal of Biological Chemistry* **274:**19403-10.

Wetzler L M. 2003. The role of Toll-like receptor 2 in microbial disease and immunity. *Vaccine* **2:**S55-60.

Williams M, Rodriguez A, Kimbrell D and Eldon E. 1997. The 18-wheeler mutation reveals complex antibacterial gene regulation in *Drosophila* host defense. *European Molecular Biology Journal* **16:** 6120.

Witte W, Tschape H, Klare I and Werner G. 2000. Antibiotics in animal feed. *Acta Veterinaria Scandavica* **93:** 45-48.

Wu X, Herndon D N and Wolf S E. 2003. Growth hormone down-regulation of Interleukin- 1beta and Interleukin-6 induced acute phase protein gene expression is associated with increased gene expression of suppressor of cytokine signal-3. *Shock* **19:**314-20.

Wyllie D H, Kiss-Toth E, Visintin A, Smith S C, Boussouf S, Segal D M, Duff G W and Dower S K. 2000. Evidence for an accessory protein function for Toll-like

receptor 1 in anti-bacterial responses. *Journal of Immunology* **165:**7125-32.

Xiao Y, Hughes A L, Ando J, Matsuda Y, Cheng J F, Skinner-Noble D and Zhang G. 2004. A genome-wide screen identifies a single beta-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. *BMC Genomics* **5:**56.

Yanagi S, Ashitani J, Ishimoto H, Date Y, Mukae H, Chino N and Nakazato M. 2005. Isolation of human beta-defensin-4 in lung tissue and its increase in lower respiratory tract infection. *Respiratory Research* **6:**130.

Yang D, Biragyn A, Kwak L W and Oppenheim J J. 2002. Mammalian defensins in immunity: more than just microbicidal. *Trends of Immunology* **23:**291-6.

Yang D, Chertov O, Bykovskaia S N, Chen Q, Buffo M J, Shogan J, Anderson M, Schroder J M, Wang J M, Howard O M and Oppenheim J J. 1999. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286:**525-8.

Yang R B, Mark M R, Gray A, Huang A, Xie M H, Zhang M, Goddard A, Wood W I, Gurney A L and Godowski P J. 1998. Toll-like receptor-2 mediates lipopolysaccharide- induced cellular signalling. *Nature* **395:**284-8.

Yarovinsky F, Zhang D, Andersen J F, Bannenberg G L, Serhan C N, Hayden M S, Hieny S, Sutterwala F S, Flavell R A, Ghosh S and Sher A. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* **308:**1626-9.

Yilmaz A, Shen S, Adelson D L, Xavier S and Zhu J J. 2005. Identification and sequence analysis of chicken Toll-like receptors. *Immunogenetics* **56:**743-53.

Yunis R, Ben-David A, Heller E D and Cahaner A. 2000. Immunocompetence and viability under commercial conditions of broiler groups differing in growth rate and in antibody response to Escherichia coil vaccine. *Poultry Science* **79:**810-6.

Zaiou M. 2007. Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. *Journal of Molecular Medicine* **85:** 317–329.

Zanetti M, Gennaro R and Romeo D. 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Letters* **374:**1-5.

Zanetti M. 2004. Cathelicidins, multifunctional peptides of the innate immunity. *Journal of Leukocyte Biology* **75:**39-48.

Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415:**389-95.

Zekarias B, Ter Huurne A A, Landman W J, Robel J M, Pol J M and Gruy E. 2002. Immunological basis of differences in disease resistance in the chicken. *Veterinary Research* **33:** 109–25.

Zhang L and Falla T J. 2004. Cationic antimicrobial peptides - an update. *Expert Opinion in Investigative Drugs* **13:**97-106.

Zhao C, Nguyen T, Liu L, Sacco R E, Brogden K A and Lehrer R I. 2001.Gallinacin-3, an Inducible Epithelial β-Defensin in the Chicken. *Infection and Immunity* **69:** 2684-2691.