MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF TOLL-LIKE RECEPTORS AND b-DEFENSINS IN AVIAN SPECIES

THESIS

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Dated: .2011

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Abbreviations

ANOVA	:	Analysis of variance
AvBD	:	Avian ß defensin
BPW	:	Buffered peptone water
CFU	:	Colony forming units
cDNA	:	Complementary DNA
Ct	:	Cycle threshold
DEPC	:	Diethylpyrocarbonate
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxynucleoside triphosphate
ED	:	Embryonic day
EDTA	:	Ethylene diamine tetraacetic acid
Fig.	:	Figure
IFN-?	:	Interferon-gamma
IL-1ß	:	Interleukin 1 beta
LB	:	Luria Bertani
mMLV-RT	:	Murine molony leukemia virus reverse transcriptase
mRNA	:	Messenger RNA
NTC	:	No template control
O. D	:	Optical density
PAMP	:	Pathogen associated molecular patterns
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PRR	:	Pattern recognizing receptors
QPCR	:	Quantitative PCR
RT-PCR	:	Reverse transcription- PCR
SDS-PAGE	:	Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis
TLR	:	Toll-like receptor

Units of Meaurement

bp	:	Base pair
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Microliter
g	:	Centrifugal force equal to gravitational acceleration
gm	:	Grams
h/hr	:	Hour
kDa	:	Kilodalton
lit	:	Liter
M	:	Molar
mg	:	Miligrams
ml	:	Milliliter
mM	:	Milimolar
rpm	:	Revolutions per miunte
min	:	Minute
V	:	Volt
nm	:	Nanometer

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1 Introduction

Animals are confronted with a wide variety of pathogens due to their microbe-rich environment. Hence, the fundamental role of immune system is to recognize the invading pathogens and to initiate an effective immune response. The immune system has traditionally been divided into innate and adaptive components, each with a different function and role. Once innate immune system was perceived as a miscellaneous collection of cells and molecules which attack microbes in a non-specific manner before the more elegant adaptive immune system takes over. However, now it is seen as a fundamental director of the overall response to infection, both as a sophisticated barrier in itself and as a potent activator and mediator of the adaptive immune response.

Adaptive immune system relies on the generation of random and highly diverse repertoire of antigen receptors: the T and B cell receptors with relevant specificities. Clonal expansion, a major mechanism involved in adaptive immune response is relatively slow, taking days before enough effector cells and their products can be produced. In contrast, the effector mechanisms of innate immunity are activated immediately after infection, with the aim to control the replication of infecting pathogen at the site of entrance. The time window of activation of antigen specific effective adaptive immune response is taken care by innate immune receptors, which provide first line defense against invading pathogens.

Innate immune mechanisms include direct bactericidal activities, such as phagocytosis, production of reactive oxygen and nitrogen intermediates (ROI and RNI respectively), secretion of antimicrobial peptides, activation of the alternative complement pathway and pro-inflammatory immune modulators (Werling and Jungi, 2003). Innate immune receptors are also known as pattern recognizing receptors (PRRs) and classified as secreted/ soluble (for example lectin binding protein, mannose binding protein) and cell associated PRRs, which include surface receptors like toll–like receptors (TLR), cytoplasmic receptors like NOD like receptors (NLRs) and RIG-1 like receptors (RLRs) (Medzhitov and Janeway, 1997; Takeda et al., 2003; Akira, 2004; Creagh and O'Neil, 2006).

These innate immune receptors/PRRs recognize highly conserved molecular structures in microbes known as pathogen associated molecular patterns (PAMPs). PAMPs include various components of pathogens such as lipopolysaccharides (LPS), peptidoglycans, flagellin, bacterial DNA and viral double stranded RNA that are shared by many pathogens but not expressed by hosts. More importantly, PAMPs are evolutionarily conserved in pathogens and critical to pathogen's function, hence cannot be eliminated or changed through mutation.

Toll-like receptors (TLRs) are important members of PRR family and recognize a wide range of PAMPs in an efficient, non self-reactive manner to initiate a complex signaling cascade to activate various transcriptional factors and pro-inflammatory cytokines. Effector mechanism of TLR pathway includes phagocytosis, production of ROI & RNI, secretion of antimicrobial peptides and activation of alternate complement pathway and finally culminate in initiation of adaptive immune response by up regualtion of co-stimulatory molecules (Medzhitov *et al.,* 1997; Takeda and Akira, 2004; West *et al.,* 2006).

Drosophila Toll (dToll) was the first member of the TLR family to be identified and was initially characterized as a developmental protein governing the formation of dorsal-ventral axis in *Drosophila* (Stein *et al.,* 1991). However, subsequent studies revealed that dToll also play a key role in triggering innate immune responses against fungal infections in adult flies (Lemaitre *et al.,* 1996). To date, 10 human and 12 murine transmembrane proteins have been shown to belong to the mammalian TLR family (Akira, 2001; Zarember and Godowski, 2002). Only in the last few years' good progress has been made in identifying TLRs in different domestic animal species including chicken.

Polymorphisms and mutations in these receptors have been implicated in disease resistance/susceptibility of chicken (Leveque *et al.,* 2003). The associations of various TLR polymorphisms with disease resistance/susceptibility will be of useful as molecular markers for selecting stocks for the improvement of disease resistance. Moreover, different TLR ligands have been shown to possess immuomodulatory effect (Taghavi *et al.,* 2008; Mackinnon *et al.,* 2009a; Dar *et al.,* 2009), hence they might be a potential future molecular adjuvant in veterinary field. Further, modulation of innate immune response of foetus by altering *TLR* gene expression by using purified/ synthetic TLR agonists have been attempted with success in various species including chicken (Kramer *et al.,* 2009; Jenkins *et al.,* 2009). In addition recent evidences indicate their extended role during embryonic development (Koga and Mor, 2010; Kannaki *et al.,* 2011).

A critical outcome of TLR stimulation is the induction of expression of antimicrobial peptides (AMPs) (Birchler *et al.,* 2001). AMPs are the direct effector molecules of the innate immune system that kill pathogens on surface through disruption of bacterial membranes (Wu *et al.,* 1999). Defensins, a subset of AMPs, are small cystein-rich cationic peptides provide a broad pathogenic defense against bacteria, viruses, fungi as well as against protozoan parasites. In addition, recent studies have proposed additional roles for these peptides including chemoattraction of immune cells and modulation of specific immune response. The immunomodulatory role exhibited by the defensin group of peptides offer strong prospects in developing them as molecular adjuvant (Tani *et al.,* 2000; Biragyn, 2005).

It has been well established that different chicken lines and strains vary in resistance/ susceptibility to diseases. *TLR* gene expression is also reported to vary among different chicken lines (Abasht *et al.,* 2008, 2009). Indigenous chicken is considered to be more disease resistant than commercial layer and broiler (Rout *et al.,* 1992). Indigenous chicken breeds are genetically distinct from other breeds (Wimmers *et al.,* 2000) having better immune competence supported by higher complement activity, higher serum lysozyme level and antibody response (Kundu *et al.,* 1999; Haunshi and Sharma, 2002; Baelmans *et al.,* 2005a). Further use of these populations in crossbreeding programs designed to create genetic stocks with improved immune status could be emphasized (Zekarias *et al.,* 2002). Immune indicators such as MHC haplotype (Baelmans et al. 2005b), cell mediated immune response, antibody immune response and serum complement levels (Dorny *et al.,* 2005; Fathi *et al.,* 2008) have been studied in native chickens, whereas no information is available regarding TLRs and defensins. Greater emphasis on egg production and weight gain by modern breeding programmes left the commercial stock immunologically underprivileged. Although chicken strains show differences in susceptibility to a number of diseases (Zekarias *et al.,* 2002) the underlying immunological basis is yet to be elucidated.

In recent years considerable attention has been given towards the immunobiology of domestic ducks (*Anas platyrhynchos*), as they are the major carriers of the highly pathogenic avian influenza virus (Webster *et al.,* 1992; Guan *et al.,* 2007). Ducks are the primary host and natural reservoir of influenza. Avian influenza H5N1strains, which are highly pathogenic to chickens rarely harm ducks (Hulse-Post *et al.,* 2005). The immunological basis for the difference in susceptibility to influenza between these two species remains to be elucidated. Moreover duck is considered as better animal models available for studying chronic hepatitis B, a major health problem of human with more than 350 million carriers existing worldwide (Blumberg, 1997). There is a paucity of information on the mediators of immune response in domestic ducks, including that of innate immunity.

Considering the all available facts and gap of knowledge in innate immune system this study was undertaken with the following objectives.

1. Expression profiling and comparative quantification of TLRs and ß defensins mRNA expression levels in a range of tissues of Indian native chicken breeds (Aseel and Kadaknath) and domestic duck (*Anas platyrhynchos*).
2. Molecular characterization of *TLR* genes of Indian native chicken breeds (Aseel and Kadaknath) and domestic duck (*Anas platyrhynchos*).
3. Functional characterization of duck TLRs by *in vitro* specific ligand stimulation studies.
4. Cloning and prokaryotic expression of avian ß defensin protein.

* * *

2

Review of Literature

2.1. Chicken toll-like receptors (TLRs)

Chicken TLR repertoire consists of ten genes namely *TLR1LA, TLR1LB, TLR2A, TLR2B, TLR3, TLR4, TLR5, TLR7, TLR15* and *TLR21* similar to that found in human and two fewer than mouse (Boyd *et al.,* 2001; Fukui *et al.,* 2001; Leveque *et al.,* 2003; Lynn *et al.,* 2003; Smith *et al.,* 2004; Iqbal *et al.,* 2005a; Philbin *et al.,* 2005; Roach *et al.,* 2005; Yilmaz *et al.,* 2005; Higgs *et al.,* 2006; Keestra *et al.,* 2007; Temperley *et al.,* 2008). Phylogenetic analyzes show these to include six orthologs of mammals and fish, one fish ortholog and three unique to chicken (Temperley *et al.,* 2008). Chicken *TLR*s *3, 4, 5* and *7* are directly orthologus to those found in other vertebrates. The chicken *TLR*s show a pattern of gene duplication and gene loss when compared to mammals. In particular, avian specific duplication of both *TLR1* and *TLR2* was observed. The duplicated genes, *TLR2A* and *2B* found in chicken are both orthologs of the single *TLR2* of mammals. Interestingly, there are remnants of second disrupted *TLR2* like gene in tandem with functional *TLR2* gene in mice and humans. Hence the duplication of *TLR2* gene might have occurred prior to the divergence of mammals and birds and subsequently lost its functionality in mammalian lineage. Chicken *TLR21* is an ortholog of *TLR21* in fish and amphibians. It appears that *TLRs 1LA, 1LB* and *15* are unique to chicken (Temperley *et al.,* 2008). The chicken genome appears to miss a number of *TLR*s which are present in most mammals. The *TLR7, 8* and *9* subfamily is present in fish and mammals but is only represented by *TLR7* in chicken (Iqbal *et al.,* 2005a; Smith *et al.,* 2004; Yilmaz *et al.,* 2005). *TLR1* and *2* underwent gene duplication in chicken, whereas *TLR8* and *9* have been lost during the course of evolution. A wide set of TLRs are expressed by immune cells such as macrophages, dendritic cells and B cells as well by non-immune cells such as epithelial cells which are located at the pathogen entry site (Iqbal *et al.,* 2005a).

2.1.1. Expression profile of chicken TLRs

TLRs are expressed by a wide variety of tissues and cell types, where they are specialized to discriminate between varieties of microbial components. Tissue, cellular localization and distribution of TLRs influence the type of immune response elicited. Most tissues express at least one TLR; many tissues express numerous TLRs (spleen and peripheral blood lymphocytes).

Chicken heterophils express *TLR1, 2, 3, 4, 5* and *7*. *TLR1, 3* and *6* are expressed in blood, kidney, liver, lung, oviduct, small intestine (SI), large intestine (LI), testis, spleen, tonsils, bursa and thymus (Kogut *et al.,* 2005a). *TLR2* was expressed in spleen, tonsils, bursa, blood, liver, oviduct and intestine (Yilmaz *et al.,* 2005; Iqbal *et al.,* 2005a). Similar to mammals, chicken monocytes and macrophages are the cell types for *TLR4* expression. *TLR4* was highly expressed in tissues rich in macrophage like cells such as spleen, tonsil and liver (Iqbal *et al.,* 2005a). In another study, *TLR4* expression was found broadly expressed in the brain, thymus, kidney, intestine, muscle, lung, liver, bursa, heart and spleen (Leveque *et al.,* 2003).

TLR5 was highly expressed in intestine, tonsils, lung, liver, kidney, spleen, heart, testis and immune cells (Iqbal *et al.,* 2005a; Leveque *et al.,* 2003). *TLR7* was found highly expressed in the lymphoid associated tissues (spleen, bursa and tonsils), lower in gut associated (SI, caecum and colon) and other tissues (thymus, bone marrow, heart, lung, liver, kidney and oviduct) and no *TLR7* expression was found in brain, muscle, testis and skin (Philbin *et al.,* 2005). *TLR15* was found abundantly expressed in bone marrow and bursa of Fabricius and moderately in spleen, liver, SI, tongue and caecum (Higgs *et al.*, 2006). Subedi *et al.* (2007) showed differential expression of TLRs by developing follicle and up regulation of *TLR4* in response to LPS administration. Chicken TLRs, nomenclature, expression profile and their ligand specificities are given in Table 2.1.

2.1.2. Molecular structure of chicken TLRs

TLRs belong to a family of type -1 transmembrane receptor characterized by an extracellular amino terminus with leucine-rich repeat (LRR) domain and an intracellular carboxyterminal tail containing a conserved region called the Toll/ interleukin-1 receptor homology domain (TIR) (Rock *et al.,* 1998). LRRs in the extracellular domain vary in number among the members, involve in ligand–binding and required for TLR dimerization upon ligand binding. The intracellular TIR domain region spans over 200 amino acids and contains three highly conserved regions (Hajjar *et al.,* 2002). The TIR domain mediates protein-protein interactions between the TLRs and signal transduction components.

Although all TLRs have similar basic structural features, mild variations have been reported. The predicted structure of chicken TLR1LB and TLR7 has been shown to have an additional transmembrane (TM) domain towards N- terminus whereas majority of TLRs have a single TM domain between the extracellular and intracellular domains (Yilmaz *et al.,* 2005; Temperley *et al.,* 2008). The predicted structure of TLR1LB is similar to that of TLR1LA except for the two missing LRRs at N-terminus.

2.1.3. Chicken TLR15

Chicken *TLR15* is avian specific and unique to chicken (Higgs *et al.,* 2006). *TLR15* gene is 2607 bp long with single exon located in chromosome no 3 (location 2945856-2948462). Although the basic structure is conserved, it has the unusual feature of many LRRs clustered towards the C-terminus of the receptor and few at the N-terminus (Temperley *et al.,* 2008). Recently Cormican *et al.* (2009) identified *TLR15* homologus to chicken *TLR15* by *in silico* analysis in Zebra finch (*Taeniapygia guttata*), a member of Passeriformes of avian lineage. *TLR15* was first identified as being up-regulated, together with *TLR2* in the cecum of chickens after infection with Salmonella (Higgs *et al.,* 2006), suggesting infiltration of cells expressing these TLRs. In addition, TLR15 was up-regulated in embryonic chicken fibroblasts after incubation with heat killed *Salmonella* spp. The agonists of TLR15 remain to be determined. However, there are substantial evidences that suggest that TLR15 recognizes components of *Salmonella* spp. (Nerren et al., 2009, 2010). Nerren *et al.* (2010) reported *TLR15* to be up-regulated in heterophils by heat killed Gram-negative or Gram-positive bacteria, but not by any of the known TLR agonists. Interestingly, heterophils isolated from broiler chickens with relative resistance to infection by Salmonella, Enterococcus, and Campylobacter demonstrated significant up-regulation of chicken *TLR15* after stimulation with live *Salmonella* spp., when compared with heterophils isolated from a susceptible line of chickens (Nerren *et al.,* 2009), suggesting TLR15 may play an important role in protection against bacterial infection in chicken. Expression of *TLR15* gene has been demonstrated in both lymphoid and non-lymphoid chicken tissues and was lower in neonates and chicken embryos (Brownlie *et al.,* 2009; Higgs *et al.,* 2006; Meade *et al.,* 2009a). However, TLR15 of other avian species are yet to identified and characterized.

2.1.4. TLRs and their ligands

TLRs recognize the specific microbial patterns and have ligand specificity. So while individually, each TLR respond to limited ligands, collectively the family of TLR can respond to a wide range of proteins associated with bacteria, viruses, fungi and parasites. Till now, ten TLRs have been identified in chicken and their ligands have been known for most of them. Chicken TLR2B was shown to act as a receptor for lipoprotein and able to recognize LPS in the presence of MD-2 similar to human TLR2 (Fukui *et al.,* 2001). TLR3 recognizes double stranded RNA (ds RNA), associated with viral infections (Alexopoulou *et al.,* 2001). HEK-293 cells expressing chTLR3 were shown to respond to poly (I:C), but not TLR7 or TLR9 agonists, using a NF?B reporter (Schwarz *et al.,* 2007), thus confirming chicken TLR3 as a receptor for TLR3 agonists. TLR4 is the principal receptor for lipopolysaccaride (LPS), which is a major component of outer membrane of gram-negative bacteria. The LPS receptor complex consists of TLR4, CD14 and MD-2 (Poltorak *et al.,* 1998). Chickens are markedly less sensitive to endotoxin delivered systemically than mammalian species (Adler and DaMassa, 1979), and it has been suggested that this may be due to absence of a TRIF/TRAM-dependent TLR4 signaling pathway in chickens; no clear orthologs to either TRAM (also known as TICAM-2) or LBP (LPS binding protein) have been identified in the chicken genome (Cormican *et al.,* 2009; Keestra and van Putten, 2008).

Chicken TLR5 recognizes flagellin, a monomeric subunit of bacterial flagella from both Gm (+) and Gm (-) bacteria as in mammals and fishes (Iqbal *et al.,* 2005b; Keestra *et al.,* 2008). TLRs 7, 8 and 9 comprise the TLR7 family that is implicated in intracellular recognition of nucleic acids. Chicken TLR7 and mammalian TLR8 have been reported to respond to R848, Poly (I:C), loxoribine and imidazoquinoline compounds with antiviral activity (Jurk *et al.,* 2002; Philbin *et al.,* 2005). Ectopically expressed chicken TLR7 in HEK-293 cells stimulated by either of the TLR7 agonists, Imiquimod or Gardiquimod, resulted in NF-?B activation (Brownlie *et al.,* 2009). Although chicken *TLR15* has been sequenced and differential expression in various tissues has been reported, ligand specificity of this TLR is still unknown. As TLR15 is reported to be avian specific and distinctly present in aves in evolutionary map their specific role needs to be explored. In mammals unmethylated CpG-DNA and synthetic oligodeoxynucleotides (ODNs) are well established ligand for *TLR9* which is predicted to be absent in chicken (Yilmaz *et al.,* 2005; Temperley *et al.,* 2008). However, similar to mammalian species chicken shows marked immunological responses to CpG -ODNs both *in vivo* and *in vitro* (Taghavi *et al.,* 2008; Mackinnon *et al.,* 2009a; Dar *et al.,* 2009). Only recently chicken TLR21 was demonstrated to act as a functional homologue to mammalian TLR9 in the recognition of CpG-ODN and bacterial genomic DNA (Brownlie *et al.,* 2009; Keestra *et al.,* 2010). However, in contrary a recent study (Ciraci and Lamont, 2011) demonstrated that rapid response to multiple classes of CpG-ODNs requires more than one specific TLR. Several receptors including TLR15, TLR21 and TLR2 respond to CpG-ODNs in a sequence-specific manner and potential cross-talk may exist among them.

Different TLRs seem to play crucial roles in the activation of the immune response to PAMPs. TLR1, TLR2, TLR4 and TLR5 tend to specialize in the recognition of bacterial components present on the outer surfaces whereas TLR3 and TLR7 specialize in recognizing nucleic acids especially of viral origin. Strategically these TLRs (3 and 7) are located in intracellular late endosomes–lysosomes compartment enabling them to differentiate host vs. pathogen nucleic acids (Kannaki *et al.,* 2010b; Brownlie and Allan, 2011). In spite of this specificity for the receptor ligand-binding, studies indicate that overall innate immune response is the sum of signals generated by the interaction of multiple TLRs.

2.1.5. Functional characterization of avian TLRs by *in vitro* studies

He *et al.* (2006, 2011) demonstrated strong induction of inducible nitric oxide synthase mRNA (iNOS mRNA) and nitric oxide (NO) production in chicken monocytes by agonists of

TLR2, 4 and 9 [lipoteichoic acid (LTA), lipopolysaccharide (LPS) and CpG-ODN respectively] by an *in vitro* study. As reported by Kogut *et al.* (2005b) TLR2 agonist like lipopeptide and peptidogycan (PGN), TLR4 agonist-LPS, TLR-5 agonist flagellin induces a significant up-regulation of IL-1ß and IL-8 when compared to non stimulated control heterophils. TLR3 agonist Poly I: C and TLR7 agonist loxoribine (LOX) induces a significant down regulation of *IL-1ß* and *IL-6*. Toll-like receptor agonist stimulates differential functional activation and cytokine and chemokine gene expression in heterophils isolated from chicken with differential innate responses (Kogut *et al.,* 2005b; He *et al.,* 2006, 2007).

2.1.6. Variation in disease resistance among chicken breeds/ lines/strains:

Variation in immune response during the course of infection is attributed to the difference in disease susceptibility (Zekarias *et al.,* 2002). It has been well established that different chicken lines and strains vary in resistance/susceptibility to diseases. TLR expression also reported to vary among different lines (Abasht *et al.,* 2009). It has been observed that TLR5 which recognizes bacterial flagellin was highly expressed in heterophils of Kadaknath chicken, whereas *TLR3* gene expression was found higher in White Leghorn in comparison with other pure lines including Dwarf, Naked neck, White Leghorn and Aseel (Kannaki *et al.,* 2010a). Significant genetic line effect was found on TLR expression in the spleen of *S. enteritidis* infected chicken which might be due to gene polymorphisms or varying number of receptors on the immune cells (Dil and Qureshi, 2002). Chicken *TLR4* gene is polymorphic and located in the genomic region associated with the resistance/susceptibility of chicks to *Salmonella enterica* serovar *Typhimurium* (Leveque *et al.,* 2003; Dil and Qureshi, 2002). The authors also demonstrated TLR4 to be required for LPS mediated *iNOS* induction in chicken macrophages. The relative number of TLR4 receptor molecules on the macrophage surface varies between chicken strains, thereby varying the expression of LPS binding receptors among strains (Dil and Qureshi, 2002). Single nucleotide polymorphisms in *TLR4* gene were identified and their association with *Salmonella enteritidis* has been established (Malek *et al.,* 2004). These results could potentially be used as markers for phenotypic variation in marker-assisted selection to enhance response to *Salmonella* spp.

Indigenous chicken is considered to be more disease resistant than commercial layer and broiler (Rout *et al.,* 1992). Indigenous chicken breeds are genetically distinct from other breeds (Wimmers *et al.,* 2000) having better immune competence supported by higher complement activity, higher serum lysozyme level and antibody response (Kundu *et al.,* 1999; Haunshi and Sharma, 2002; Baelmans *et al.,* 2005a, 2005b). Further use of these populations in crossbreeding programs designed to create genetic stocks with improved immune status could be emphasized. Broader expression profile of TLR repertoire has been reported only in White Leghorn birds implying their potential role in pathogen recognition (Iqbal *et al.,* 2005a; Kogut *et al.,* 2005a).

2.1.7. Chicken toll-like receptors gene expression during microbial infection

The expression levels of TLRs in chickens after bacterial and viral challenge have been reported by many studies. Up regulation of chicken *TLR2B*, *TLR3* and *TLR15* mRNA in the caecum of *Salmonella enterica* serovar Typhimurium infected chicken and in chicken embryonic fibroblasts following stimulation with heat-killed *Salmonella enterica* serovar Typhimurium have been reported (Higgs *et al.,* 2006). Interestingly, down regulation of *TLR5* gene expression was observed after Salmonella infection (Iqbal *et al.,* 2005b; Abasht *et al.,* 2008) in chicken gut epithelial cells, most likely attributed to negative feedback needed to limit overstimulation in an area of high bacterial colonization. The gene expressions of *TLR4* and *TLR7* were up regulated in spleen of chickens challenged with *Clostridium perfringens* (Lu *et al.,* 2009) but could not appreciate significant changes in myeloid differentiation factor 88 (*MyD88*) or tumour necrosis factor receptor-associated factor 6 (*TRAF6*) pathway molecules quantified by real-time PCR. The authors have hypothesized that chicken TLR may not signal through *MyD88* pathway as TLR2 do in mammals, which is contrary to the highly conserved nature of innate immune system. The gene expression of *TLR3* and *TLR7* was enhanced in response to Marek’s disease virus infection in the chicken lungs (Abdul-Careem *et al.,* 2009). *TLR1* gene expression was decreased and avian beta-defensin (*AvBD*) gene expression (*AvBD3*, *AvBD10* and *AvBD12*) was significantly increased in response to *Salmonella* infection. In contrast, Campylobacter infection increased *TLR21* gene expression but significantly reduced the expression of seven antimicrobial peptide (AMP) genes (*AvBD3*, *AvBD4*, *AvBD8*, *AvBD13*, *AvBD14*, Cathelicidins 2 and 3) (Meade *et al.,* 2009b). Significant increase in *TLR5* and *TLR15* gene expression was detected in response to *S*. Typhimurium but not to *C*. *jejuni*. *TLR4* and *TLR21* gene expression were transiently increased in response to both bacterial species. The differential expression profile of innate immune genes in both infection models shed light on the tailored responses of the host immune system to specific microbes (Shaughnessy *et al.,* 2009). TLR7 mediated *IL-4* secretion was reported in chickens infected by low-pathogenicity avian influenza virus H9N2 (Xing *et al.,* 2008).

After infection of broiler chickens with *Clostridium perfringens*, increased expression was observed for the TLR2-family, *TLR4*, *TLR15* and *TLR21*, as well as for several TLR adaptor molecules in the spleen and ileum (Lu *et al*., 2009); significant increases of *TLR5* and *TLR15* gene expressions were observed in the caecum of chickens after infection with Salmonella (Shaughnessy *et al.,* 2009); *TLR3* and *TLR7* gene expressions were up-regulated in the lungs of chickens after infection with Marek's disease virus, and this was associated with up-regulation of pro-inflammatory cytokines and macrophage infiltration (Abdul-Careem *et al.,* 2009). However, these results may reflect infiltration or proliferation of cells expressing these TLRs.

2.1.8. TLRs of other avian species

Most of the understandings about the avian TLR family are solely based on chicken TLR family. Only few TLRs of other avian species (TLR4 of zebran finch, TLR7 of duck) have been molecularly characterized (MacDonald *et al.,* 2008; Vinkler *et al.,* 2009). Philbin *et al.* (2005) showed that disruption of *TLR8* gene was found in galliform bird species including Red jungle fowl, guinea fowl, Japanese quail, pheasant and turkey, whereas non-galliform bird species like Pekin duck, goose, black swan, penguin and ostrich did not possess the CR1 element tested by nested PCR. Hence, it was concluded that disruption of *TLR8* gene only in galliform species might contribute to the difference in viral susceptibility. In contrast a recent study (MacDonald *et al.,* 2008) examined *TLR8* gene organization of Pekin duck, a non-galliform bird species by sequencing genomic clone spanning the TLR7/8 locus and detected only fragments of *TLR8* gene and demonstrated insertion of CR1 element between this gene fragments. Further studies are warranted to explore *TLR8* gene organization in other non-galliform bird species. Both *TLR7* and *8* are implicated in the detection of single stranded viruses in mammals. TLR7 may be involved in detection of influenza virus in birds in the absence of functional TLR8. The critical difference in susceptibility to influenza virus between ducks and chicken remains to be identified. Only *TLR7* and *TLR8* have been investigated in domestic duck in a preliminary study (MacDonald *et al.,* 2008). Other TLRs and their expression profile have to be investigated. Recently TLR repertoire has been identified by *in silico* analysis in singing bird's (zebra finch) genome. Despite being distantly related to the chicken, analysis of zebra finch genome showed overall conformity of TLR family and downstream signalling components (Cormican *et al.,* 2009). Recently whole genome of turkey has been sequenced and available in public domain (Dalloul *et al.,* 2010). Draft genome of duck is available in Pre-Ensemble genome database (unpublished).

2.2. ß-Defensins

Defensins are a group of small cationic peptides that play an important role in the innate immune system of vertebrates and invertebrates (Wong *et al.,* 2007). Defensins constitute a large family of small, cysteine-rich, cationic peptides that are capable of killing a broad spectrum of pathogens, including various bacteria, fungi, and certain enveloped viruses. The avian antimicrobial activity of heterophils depends mainly on oxygen-independent mechanisms such as cationic antimicrobial peptides and lysozyme as they lack myeloperoxidase, an essential enzyme for respiratory burst (Harmon *et al.,* 1992).

In addition to their direct antimicrobial activities, immunomodulatory properties have also been demonstrated. Defensins can promote adaptive immunity by selective recruitment by chemotaxis of monocytes (Territo *et al.,* 1989), T lymphocytes (Chertov *et al.,* 1996), immature dendritic cells (Yang *et al.,* 1999) and mast cells (Niyonsaba *et al.,* 2002) to the sites of inflammation.

In the chicken genome, 14 genes coding for ß-defensins have been found in a dense cluster located on the end of the 3rd chromosome (3q3.5-q3.7) (Lynn *et al.,* 2004; Xiao *et al.,* 2004). These genes consist of four exons (E1-E4), E1 coding for 5' UTR, E2 the signal peptide and a part of the propiece (i.e. the part of the prepeptide that is later cleaved off to get the mature peptide), E3 codes for the rest of the propiece and the mature peptide and the fourth exon codes for the 3'-UTR. ß-defensins are expressed and excreted by neutrophils and epithelia cells lining various organs (van Dijk *et al.,* 2008).

2.2.1. Expression profile of ß defensins in chicken:

In the chicken, the tissue-specific pattern seems to vary across the different defensin genes with some showing expression in a wide array of tissues (e.g. *AvBD9* expression was found in 22 different tissues, ranging from brain to different intestines and testis) (van Dijk *et al.,* 2008) whereas other seem to have more limited expression patterns (e.g. *AvBD8* with expression only observed in the liver and gall-bladder).

AvBD4–7 are strongly expressed in bone marrow, whereas weak or no mRNA expression was found for AvBD*4–7* in heterophils (Xiao *et al.,* 2004; Lynn *et al.,* 2007). In the respiratory tract, high ß-defensin expression was observed for *AvBD3* (Zhao *et al.,* 2001) and *AvBD9* (van Dijk *et al.,* 2007) in trachea. In lung tissue moderate to strong expression was found for *AvBD1* and *2* (Lynn *et al.,* 2004; Zhao *et al.,* 2001). In the proximal digestive tract, strong expression was observed for *AvBD3* and *5* (Lynn *et al.,* 2004) in tongue and *AvBD9* (van Dijk *et al.,* 2007) in esophagus and crop tissue. The high expression levels of *AvBD9* in adult chicken crop tissue and its variable expression in juvenile broilers indicate an important role of *AvBD9* in crop tissue defense (van Dijk *et al.,* 2007). Developmental expression studies of chicken *AvBD4* mRNA using 1, 4, 17 and 38-day-old animals showed *AvBD4* expression to be maximal within the first week post-hatch and to decline thereafter (Milona *et al.,* 2007). Considerable *AvBD13* mRNA expression was found in small intestinal tissue, liver and gall bladder (Higgs *et al.,* 2006). Similarly, moderate to high mRNA expression in liver (and gall bladder) was found for *AvBD8, 9,* and *10* (Higgs *et al.,* 2006; Lynn *et al.,* 2004; Xiao *et al.,* 2004) which may reflect an important role of avian ß-defensins in the liver during systemic infections. Moderate *AvBD1* and *2* expression was observed in caecal tissue of 3–7-week-old (Sadeyen *et al.,* 2004) and 30-week-old chickens (Sadeyen *et al.,* 2006). The contrast in ß-defensin expression levels between bursa of Fabricius, spleen and thymus, bursa>>spleen > thymus might be due to their location (van Dijk *et al.,* 2008).

2.2.2. ß-defensins of other avian species

Recently, ß-defensin-2 homologue from domestic ducks has been identified from the cDNA library constructed by subtraction cloning technique. Further antibacterial activity against both Gram-positive and Gram-negative bacteria, chemotactic activity on DT-40 cells and immunomodulatory activity has been demonstrated (Soman *et al.*, 2009a, 2009b; Ma *et al.*, 2009a,b). ß-defensins are also identified in turkey (Evans *et al.*, 1994), ostrich (Sugiarto and Yu, 2006) and king penguin (Thouzeau *et al.*, 2003).

2.2.3. Structural features of avian defensins

Deduced primary amino acid sequences for avian mature defensins indicate that, similar to mammalian ß defensins (Pazgier *et al.*, 2006), these peptides consist of 36 aa or more residues with the consensus sequence motif: xn-C-x2–4-G-x1–2-C-x3–5-C-x9–10-C-x5–6-CCxn. The overall fold consisted of a three-stranded β-sheet and an α helical N-terminus in the structure and contained a hydrophobic patch (Phe^{19}-Pro^{20}-Ile^{22}-Val^{37}-Trp^{38}), shown by comparative structure analysis to be well but not strictly conserved in other avian defensins. Some avian ß-defensin genes, e.g. *AvBD3*, *11* and *13*, contain a large postpiece, although the *AvBD13* nucleotide sequence reported by Higgs *et al.* (2005) lacks this postpiece suggesting that there might be strain-specific splice variants or isoforms of the *AvBD13* gene (Higgs *et al.*, 2005). Comparative analysis of the nucleotide sequences of chicken and turkey *AvBD3* (91% identical on a nucleotide level), the latter of which lacks a postpiece, revealed that a two-base insertion just before the chicken *AvBD3* original stop codon, causes a frame shift, and an additional 15 bp insertion was responsible for the generation of an anionic postpiece in chicken AvBD3 (Zhao *et al.*, 2001).

2.2.4. Cloning and expression of avian defensins

Although initially expression of the antimicrobial proteins in recombinant form in prokaryotic system was considered difficult due to their toxicity to bacterial cells (Piers *et al.*, 1993), recently some researchers demonstrated the recombinant expression in prokaryotic system. Chicken defensins 8 and 9 has been cloned and expressed in *E. coli* as inclusion bodies (Ma *et al.*, 2008). Higgs *et al.* (2007), cloned and expressed *AvBD8* in *E. coli.*

Recently Soman *et al.* (2009a) expressed duck defensin-2 in both prokaryotic and mammalian cell culture system.

2.2.5. Antimicrobial activity of avian defensins

The most distinct molecular feature of defensins is their high pI value, ranging from +6 to +12 as monomers, manifested by abundant arginine and lysine residues in their sequences. They kill microorganisms through permeabilization of the microbial membrane composed of negatively charged components such as phospholipids, teichoic acids and lipopolysaccharides. It is believed that electrostatic interactions dictate not only the uptake of cationic defensins across the bacterial cell wall but also their ability to permeabilize the cytoplasmic membrane and to induce leakage of cellular contents (van Dijk *et al.*, 2008).

Evans *et al.* (1995) demonstrated bactericidal and fungicidal activity of chicken and turkey heterophil AvBD1, at peptide concentrations of 0.4–3.4 µM and 0.4–1.8 µM, respectively, against avian pathogens. However, these peptides were not able to kill *P. multocida* or neutralize infectious bronchitis virus, an enveloped coronavirus of chickens. A (20 aa) fragment of turkey AvBD2 inhibited the growth of *S. aureus*, but not of *E. coli* (Evans *et al.*, 1994). Synthetic chicken AvBD9 peptide showed strong microbicidal activity against the Gram-negative bacterium *C. jejuni* (3.7 µM), Gram-positive bacteria, *C. perfringens*, *S. aureus* (1.9–3.7 µM) and the yeasts *C. albicans* and *S. cerevisiae* (1.9 µM), but was less potent against *E. coli* (7.5 µM) and not bactericidal against *S.* Typhimurium (>30 µM) (van Dijk *et al.,* 2007). In contrast, synthetic chicken AvBD13 peptide was only bactericidal at high peptide concentrations against *L. monocytogenes* (114 µM) and *S*. Typhimurium wild-type (114 µM) and a *S*. Typhimurium Pho P mutant (57 µM), whereas inhibition of *E. coli*, *S. aureus* and *S. pyogenes* at peptide concentrations of 57 µM was negligible or absent (Higgs *et al.*, 2005).

In radial diffusion assays, ostrich heterophil ß-defensins, AvBD1, 2, and 7, efficiently inhibited the growth of *E. coli* O157:H7 and methicillin-resistant *S. aureus* strain 1056 (MRSA) with minimum inhibitory concentrations (MIC) ranging from 0.2 to 0.6 µM (Sugiarto and Yu, 2006). Ostrich AvBD8 was less potent against these bacterial strains (MIC, 2.4 µM), whereas only Ostrich AvBD1 was fungicidal against *C. albicans*.

Analysis of the stomach contents of male king penguins revealed numerous antimicrobial activities, including the avian ß-defensin peptides AvBD103a and AvBD103b, which are identical with the exception of an Arg residue instead of a His residue at position 14 for AvBD103b. Synthetic penguin AvBD103b peptide displayed potent bacterical activity against Gram-positive bacteria (*K. rhizophilae, Bacillus* spp., *Staphylococcus* spp., *N. asteroides* and *A. viridans*), with the exception of *S. saprophyticus,* at peptide concentrations less than 4 µM (Thouzeau et al., 2003). Mainly bacteriostatic activity was observed for AvBD103b against Gram-negative bacteria, although it displayed bactericidal activity against an *E. coli* strain. In contrast to its impotence against *Candida glabrata* (>100 µM) and *Candida albicans* (50–100 µM), the yeast *Candida tropicalis* and filamentous fungi *Neurospora crassa* and *Aspergillus fumigatus* were efficiently killed (3–6 µM) by AvBD103b.

* * *

3 Materials and Methods

3.1. Materials

3.1.1. Chemicals

Standard molecular biology grade and analytical grade chemicals and reagents procured from Merck (Germany), SRL (India), Qiagen (Switzerland), Ambion (USA), Amresco (USA), Promega (USA), Sigma-Aldrich (USA), MBI Fermentas (USA), Chromous biotech (India) and Bangalore Genei (India) were used.

3.1.2. Glassware and Plastic ware

All the glassware used for this study were from Borosil (India), Duran Scott (Germany) and Corning (USA). They were thoroughly washed and sterilized as per standard procedure. All the plasticware were purchased from Axygen (USA) Nunc (Denmark) and Tarsons (India). All the plasticware employed for RNA isolation and cDNA preparation were treated overnight with 0.1% Diethyl pyrocarbonate (DEPC) (MBI Fermentas, USA).

3.1.3. TLR agonists

Two commercially available TLR agonists, LPS from *E. coli* serotype O26: B 11 (Sigma-Aldrich, USA) and poly I: C, a synthetic dsRNA analog (Invivogen, USA) were used in this study.

3.1.4. Primers

All the primers used in this study were custom synthesized from IDT (USA) and ILS (India). Gene specific primers used in this study have been presented under appropriate sections.

3.1.5. Enzymes

Proteinase-K (Sigma-Aldrich, USA), RNase free DNase I (MBI Fermentas, USA), Taq DNA polymerase (MBI Fermentas, USA), reverse transcriptase M-MuLV (MBI Fermentas, USA), restriction enzymes viz., Nde I, Xho I, EcoRI and Hind*III* (MBI Fermentas, USA) were used in this study.

3.1.6. Experimental birds

Day old chicks of Indian native breeds Aseel and Kadaknath and inbred White Leghorn and broiler (PB-1) breeds obtained from Project Directorate on Poultry, Hyderabad were used in the study. Day old ducklings, turkey poults and 3-month-old Japanese quails were purchased from Poultry science department, College of Veterinary Science, Hyderabad. All the chicks were maintained under standard conditions. All experiments were carried out with the approval of Institute Animal Ethics Committee.

3.1.7. Fertile chicken eggs

Fertilized White Leghorn eggs were obtained from hatchery of Project Directorate on Poultry, Hyderabad and incubated at 37 ± 0.5^{o}C with 70-80% relative humidity.

3.1.8. Bacterial strains

Salmonella enterica serovar Pullorum, virulent field isolate, was obtained from the National Salmonella Center, Indian Veterinary Research Institute, India. *Escherichia coli* strains DH5a (Promega, WI) and BL21 (DE3) (Promega, WI) were used for cloning and recombinant protein expression studies.

3.1.9. Media and Buffers

Brilliant Green agar (Himedia, India) Luria Bertani (LB) broth and agar (Himedia, India), RPMI-1640 growth medium (Sigma-Aldrich, USA) and newborn calf serum (Invitrogen, USA) were used. The composition of media and buffers used in this study are given in Appendix.

3.1.10. Cloning and expression vectors

Cloning vector, pUC29 (Invivogen, USA) and expression vector, pET28a (Novagen, WI) were used in the present study.

3.1.11. Software

Softwares such as DNA Star (Lasergene, USA), MEGA 4.0 (Tamura *et al.,* 2007), CLUSTAL W (Larkin *et al.,* 2007) and online free tools such as SMART program (http://smart.embl-heidelberg.de), TMHMM (Letunic *et al.,* 2004; http://www.cbs.dtu.dk/service/TMHMM/), SignalP program (www.cbs.dtu.dk/service/SignalP) and BLASTP (http://www.ncbi/blastP) were used. Draft genomes of duck (duck1) (http://pre.ensembl.org/*Anas_platyrhynchos*/Info/Index) and turkey (UMD 2.0) genomes (http://ensembl.org/*Meleagris_ gallopavo* /Info/Index) and gene sequences from NCBI were used.

3.2. Methods

3.2.1. Standard molecular techniques used in this study

3.2.1.1. RNA isolation

Total RNA was extracted from each tissue sample (50mg) using Trizol reagent (Invitrogen, USA), according to the manufacturer's instruction. Briefly the steps are as follows:

- 50mg of tissue sample was finely crushed and 1 ml of Trizol reagent was added and kept at room temperature for 10-15 min.
- Phase separation: 0.2 ml of chloroform was added and mixed vigorously for 10-15 seconds, kept at room temperature for 2-3 min and then centrifuged at 12000 g for 15 min for phase separation. After centrifugation two separate layers, upper aqueous phase and lower protein and Trizol phase were formed.
- RNA precipitation: The aqueous colourless phase of the solution was collected into a new DEPC treated eppendorf tube and to that 0.5 ml of isopropanol was added to precipitate the RNA by centrifugation at 12000 g for 10 min.
- RNA washing: After precipitation, RNA was washed with 1 ml of 75% ethanol by centrifugation at 7000 g for 5 min.

- Drying: After washing, the precipitate was air dried and then dissolved in 25 µl of nuclease free water. To avoid the possible traces of genomic DNA, 5 µg of each RNA sample was incubated at 37^{o}C for 10 min with 5 U of RNase free DNase I (MBI fermentas, USA), following this step DNase was inactivated by incubation at $65^{?}$C for 10 min.
- An aliquot of the total RNA was subjected to denaturing agarose gel (1%) electrophoresis to assess the quality and integrity of the RNA.
- The purity and concentration of samples were checked using the Nanodrop Spectrophotometer (ND 1000, Thermo Scientific, USA) by taking reading at OD_{260} and OD_{280} taken against 1µl nuclease free water as blank. The RNA samples that showed the $OD_{260}/OD_{280} = 2$ were free of protein contamination. The concentration of total RNA was calculated as follows:

$$\text{RNA concentration } (\mu g/\mu l) = OD_{260} \times \text{(Dilution factor)} \times 40/1000$$

3.2.1.2. cDNA preparation

First strand cDNA was synthesized from RNA samples using oligo (dT) primer and MuMLV reverse transcriptase (MBI Fermentas, USA) in a 20 µl reaction mixture as follows:

Total RNA (1 µg)	3 µl
Oligo dT_{24} (50 µM, 80 µl, Ambion, USA)	1 µl
RNase free water (Qiagen, Germany)	6 µl

The above mixture was incubated 70^{o}C for 5 min, snap cooled on ice and then following reagents were added.

5X reverse transcription buffer	4 µl
Superasein (RNase inhibitor, 200IU/µl Ambion, USA)	1 µl
10 mM dNTP mix	2 µl

The mixture was incubated at 37^{o}C for 5 min and then following enzyme was added.

Revert Aid M-MuLV-RT (200U/µl, MBI Fermentas, USA)	2 µl

Finally incubated at 42°C for 60 min followed by 70°C for 10 min and the cDNA was stored at -20°C till further analysis.

3.2.1.3. PCR amplification of ß-actin using gene specific primer (cDNA checking)

The cDNA prepared was checked by PCR reaction with chicken ß-actin specific primers in a reaction using 10 pmol of each primer and 1µl of cDNA template. Thermal profile consisted of an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30s; annealing at 55°C for 30s and extension at 72°C for 30s. The amplified products were checked in agarose gel. Briefly, 5 µl of each PCR product was analyzed in 1.25% agarose gel containing ethidium bromide (0.5 µg/ml). The gels were run at 80V for 45-60 min and visualized under UV illuminator (UVP, USA).

3.2.1.4. Quantitative analysis of gene expression by real-time PCR

Relative quantification of gene expression in tissue samples was done by real-time PCR using the Mx3000P QPCR system (Stratagene, USA). The amplification was performed in a total volume of 25 µl, containing 1X QuantiTect SYBR Green PCR master mix (SYBR Green I dye, ROX passive reference dye, HotStartTaq DNA polymerase and dNTPs with dUTPs in optimized buffer, Qiagen GmBH, Germany), 10 pmol of each gene specific primer and 0.5 µl of cDNA template. All gene specific primer sequences have been specifically mentioned in respective sections. Thermal profile consisted of an initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30s; gene specific annealing temperature (55-60°C) for 30s and extension at 72°C for 30s. Dissociation analysis of amplification products was performed at the end of each PCR to confirm the specificity of amplicon. In each PCR reaction no template control (NTC) was included to check contamination of master mix. Non-reverse transcribed RNA (10 ng) of each sample was used instead of cDNA to check contamination of samples with genomic DNA, failure of amplification confirms the purity of sample.

3.2.1.5. Efficiency of primer pairs in real-time PCR assay

To assess the efficiency of primers, standard curves for each primer pair were generated using serially diluted transcribed RNA sample. PCR efficiency was calculated from the slope of standard curves. The resulting threshold cycle (Ct, a fractional PCR cycle number at which the change in reporter dye (DRn) passes the significant threshold) values were normalized to the endogenous control, ß actin (ΔCt = Ct value of target gene-Ct value of ß actin).

3.2.1.6. Statistical analysis

Statistical models such as student‘t’ test, one way ANOVA, two way ANOVA were employed for analysis. All analyses were done in SPSS 10.0 (SPSS Inc, Chicago, IL) and SAS 9.2 version. Details of analysis have been given under each section.

3.2. Comparative quantification of constitutive TLRs mRNA expression in Indian native and White Leghorn chickens

3.2.1. Tissue sample collection

Six day-old chicks each from native chicken breeds (Aseel and Kadaknath) and White Leghorn were sacrificed by cervical dislocation. Samples from a range of lymphoid and non-lymphoid tissues such as heart, liver, spleen, intestine, bursa, bone marrow and skeletal muscle were aseptically collected and processed immediately.

3.2.2. Quantitative analysis of TLRs mRNA by real-time PCR

Total RNA was extracted from each tissue sample using Trizol and first strand cDNA was synthesized as per section 3.2.1.1and 3.2.1.2 respectively. Relative quantification of chicken TLRs (*3, 4, 5, 7, 15* and *21*) in tissue samples was done by real-time PCR using the Mx3000P QPCR system (Stratagene, USA). The chicken TLR gene specific primer sequences are presented in Table 3.1.

3.2.3. Statistical analysis

To convey the inverse relationship between starting template concentration and Ct value, results were expressed and analyzed as 40-ΔCt values, interpreted as higher 40-ΔCt value implying greater gene expression. Analysis of 40-?Ct mean values was carried out using two-way ANOVA with Tukey’s post-hoc test using SPSS 10.0.1 (SPSS Inc, Chicago, IL) to identify significant difference in gene expression between different tissues among the chicken breeds. Values were considered significant at $P<0.05$.

Table 3.1. Chicken TLR gene specific real-time PCR primer sequences used in this study

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing Temp.(^{o}C)	Amplicon size	Accession no.
TLR1LA	TTACTGCCAATTGCTTGCAC	GGTTAGGAAGACCGTGTCCA	56	187	AY633574
TLR1LB	CCCGTTCAAGTGTTCATGTG	GTTCCGCTCAAGTCTTCTGG	57	120	NM001081709
TLR2A	ACATGTGTGAATGGCCTGAA	TTGAGAAATGGCAGTTGCAG	56	151	NM 204278
TLR2B	TTCGCTCCAACACCTTCG	CTGATGACTGCTGAAATACG	58	279	NM 001161650
TLR3	AGACACAGCAATTCAGAAC	TTAATGATGTTATTATCCTCCAAG	55	198	NM_001011691
TLR4	CCATCCACTCAGACAACCTTTCCA	AGTAAACGCAGCAGACGCG	55	219	AY064697
TLR5	CACTGCTGGAGGATTTGTTCTTG	ACAGACGGAGTATGGTCAAACG	55	183	NM_001024586
TLR7	GATGCAGTGTGGTTTGTTGG	AACCAAGCTCCTCCCTTTGT	56	111	NM 001011688
TLR15	TCTTCTGGTATCTGGTCTTGC	CCTGGATTGGGTGGATCTTC	55	165	NM_001037835
TLR21	AGCTGGAGCTGTTGGACCTA	TTCACGTGCCATAGCATCTC	56	148	NM 001030558
ß actin	GCACCACACTTTCTACAATGAG	ACGACCAGAGGCATACAGG	55	184	L08165

3.3. Differential *TLR*s mRNA expression patterns during chicken embryological development

3.3.1. Sample collection

Tissue samples were collected on third and fifth embryonic day (ED) and at regular interval afterwards (7, 9, 12, 15 and 18 ED) aseptically at the same time each day. Whole embryos were collected on 3rd and 5th ED (n=4/each day) and tissues from various organs (liver, heart, brain and intestine) were collected from 7 ED (n=4/each day) onwards. All tissue samples were kept at -80°C till further processing.

3.3.2. Relative quantification of Toll-like receptors mRNA by real time PCR

Total RNA was extracted from each chicken embryo tissue sample using Trizol as described in section 3.2.1.1. First strand cDNA was synthesised as per section 3.2.1.2. Relative quantification of chicken TLRs (*2A, 3, 4, 5, 7, 15* and *21*) in tissue samples was done by real-time PCR as described in section 3.2.1.4.

3.3.3. Statistical analysis

The fold change in TLR gene expressions during chicken embryonic development in different tissues were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and expression levels for respective TLR gene at 3 ED tissues were used as calibrators. Statistical comparisons of fold changes in gene expression in different tissues and on different EDs were performed using General Linear Model in version 9.2 of SAS software with a P value <0.01 considered significant.

3.4. Comparative quantification of constitutive ß-defensins mRNA expression in Indian native and White Leghorn chickens

A total of six day-old chicks from each Indian native breeds (Aseel and Kadaknath) and White Leghorn breed were randomly chosen and were euthanized. Spleen tissue from all chicks were aseptically collected and processed immediately. Total RNA was extracted from each sample using Trizol as described in section 3.2.1.1. First strand cDNA was synthesised as per section 3.2.1.2. The constitutive expressions of mRNA of *AvBD*s (*1-14*) were quantified

Table 3.2. Chicken *AvBD* genes (*1-14*) specific real-time PCR Primers used in this study

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
AvBD1	CGAAAGAGTGGCTTCTGTGC	GGTGATGTCCTGCTTGGG	156
AvBD2	AGGTTTCTCCAGGGTTGT	TGCATTCCAAGGCCATT	146
AvBD3	CCACTCAGTGCAGAATAAGAG	AATTCAGGGCATCAACCTC	131
AvBD4	CATCTCAGTGTCGTTTCTCTGC	CGCGATATCCACATTGCAT	157
AvBD5	CTGCCAGCAAGAAAGGAACCTG	GTAATCCTCGAGCAAGGGACA	155
AvBD6	AGGATTTCACATCCCAGCCGTG	CGACATGGCCCAGGAATGCA	156
AvBD7	TGGAGAAGGGAGACAGAAGGCA	CGAAGCCTACAAGTATCAAT	177
AvBD8	ACAGTGTGAGCAGGCAGGAGGGA	GAAGAGCTGCTTAGCTGGTC	153
AvBD9	GCAAAGGCTATTCCACAGCAG	GGAGCACGGCATGCAACAA	167
AvBD10	TGGGGCACGCAGTCCACAAC	CATGCCCCAGCACGGCAGAA	157
AvBD11	ACTGCATCCGTTCCAAAGTCTG	GTCCCAGCTGTTCTTCCAG	168
AvBD12	CCCAGCAGGACCAAAGCAATG	AGTACTTAGCCAGGTATTCC	157
AvBD13	CATCGTTGTCATTCTCCTCCTC	GGTGGAGAACCTGCAGCAGCG	163
AvBD14	ATGGGCATATTCCTCCTGT	CACTTTGCCAGTCCATTGT	161

by SYBR green method by using Mx-3000P spectrofluorometric thermocycler (Stratagene, USA) as per section 3.2.1.4. Primer pairs specific for the amplification of *AvBD* genes (Ebers *et al.*, 2009) are given in Table 3.2. The chicken ß actin gene was used as an endogenous control. Primer amplification efficiencies were assessed for each gene from the standard curve generated by using serial tenfold dilution of transcribed RNA. Analysis of 40-?Ct mean values was carried out using one-way ANOVA with Tukey's post-hoc test using SPSS 10.0 (SPSS Inc, Chicago, IL) to identify significant difference in gene expression among the chicken breeds. Values were considered significant at P<0.05. Fold change in *AvBD* gene expression in native chicken breeds were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using White Leghorn as calibrator.

3.5. Differential innate immune gene expressions in the gastrointestinal tract of chicken upon *S*. Pullorum-infection

3.5.1. Experimental birds

Day-old-broiler chicks were obtained from Project Directorate on Poultry, Hyderabad, India and were reared in experimental facility of Microbiolgy division, College of Veterinary Science, Hyderabad. Birds were given *ad libitum* access to water and feed. Chicks were confirmed for free status of Salmonella by culturing faecal samples in buffered peptone water (BPW) overnight and by spreading on brilliant green agar containing 100 µg/ml nalidixic acid.

3.5.2. Experimental infection with *S.* Pullorum and sample collection

Salmonella enterica serovar Pullorum was grown overnight in Luria-Bertani (LB) broth at 37°C in an orbital shaking incubator at 150 rpm. The bacteria were washed three times by pelleting at 10,000 x g for 10 min and resuspended in PBS. The concentration of suspension was determined by following standard plate count technique by plating serial dilutions of bacterial suspension and adjusted to 10^8 CFU/ml. Chickens were randomly divided into two groups of 12 each at 3 days of age. One group was orally inoculated with 0.5 ml of the bacterial suspension containing 10^8 CFU/ml and the control group was given 0.5 ml of PBS. A total of six chickens from each group were randomly chosen and were euthanized 24 h post-challenge. The entire GI tract was removed aseptically and a 50 mg piece of each tissue was removed from the middle of the duodenum (D), jejunum (J), ileum (I) and caecum (C). The tissues were washed in PBS and placed in a microcentrifuge tube containing 1ml Trizol and immediately processed. In addition, swabs from caecum of both groups were taken and cultured in BPW overnight and streaked on brilliant green agar plates containing 100 µg/ml nalidixic acid (37°C for 18-24h) to confirm that control birds were uninfected and the challenged chicks were infected.

3.5.3. Quantitative analysis of *TLR*s and *AvBD*s mRNA by real-time PCR

Total RNA was extracted from each sample using Trizol as described in section 3.2.1.1. First strand cDNA was synthesised as per section 3.2.1.2. Relative quantification of chicken TLRs (*TLR1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15* and *21*) and *AvBD* (*1-14*) genes in GI tissues was done by real-time PCR as described in section 3.2.1.4.

3.5.4. Statistical analysis

All expression data are relative mRNA levels presented as mean 40-ΔCt values ±SEM. Student's t-test for comparison of two independent samples was used to evaluate differences between the infected and uninfected groups. Values of $P<0.05$ were considered statistically significant. For presentation purpose the fold-changes are calculated as $2^{\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). However, statistical analysis was not performed on the fold-changes but only performed on the individual 40-ΔCt values for each TLR.

3.6. Identification, molecular characterization and expression analysis of duck and turkey TLR genes

3.6.1. *In silico* identification of TLR genes in duck and turkey genome

A dataset of all known TLR proteins of chicken (Temperley *et al.*, 2008) and zebra finch (Cormican *et al.*, 2009) was created by downloading the sequences from NCBI (http://www.ncbi.nlm.gov) and Ensembl (http://ensembl.org/Taeniopygia_guttata/info/index) databases respectively. The accession numbers of the sequences used for gene-searching and phylogenetic analysis are listed in Table 3.3. The draft version of sequenced and assembled duck (duck1) and turkey (UMD 2.0) genomes available at (http://pre.ensembl.org/*Anas_platyrhynchos*/Info/Index) and (http://ensembl.org/*Meleagris_ gallopavo*/Info/Index)

Table 3.3. The accession numbers of TLR genes used for BLAST and phylogenetic analyses

TLR genes	Chicken	Zebra finch	Duck	Turkey
TLR1LA	BAD67422	ENSTGUP00000009313	ACS92621	FJ477857
TLR1LB	ABF67957	XP_0021897591	ACS92622	FJ477858
TLR2A	NP_989609	XP_002196402	ACS92627	FJ477860
TLR2B	BAB16842	XP_002198506	ACS92628	FJ477861
TLR3	NP_001011691	XP_002190888	-	-
TLR4	AAL49971	NP_001135926	-	-
TLR5	CAF25167	XP_002188762	-	-
TLR7	NP_001011688	XP_002194911		
		XP_002194932*	DQ888645	-
TLR15	NP_001032924	XP_0021971051	-	-
TLR21	NP_001025729	Pseudogene	-	-

The accession numbers of NCBI protein database are listed *TLR7-1, TLR7 gene is duplicated in zebra finch genome were used for analysis. NCBI nucleotide database were searched with BLASTP program for the orthologus gene members of assembled TLR gene data set. Genes showing high degree of similarity to avian counterparts were selected and their corresponding nucleotide and amino acid sequences were retrieved and analyzed. Domain structures of the turkey TLR proteins were analyzed by the SMART program (http://smart.embl-heidelberg.de).

3.6.2. Phylogenetic and evolutionary analysis

An unrooted phylogenetic tree based on the amino acid sequences was constructed by the Neighbor-joining (NJ) method in the Clustal X version 2 program (Larkin *et al.,* 2007) and the MEGA version 4 program (Tamura *et al.,* 2007). The distance matrix was obtained by calculating p-distances for all pairs of sequences. Sites containing gaps were excluded from the analysis using the pair wise deletion option. The reliability of branching patterns was assessed by bootstrap analysis (1000 replications). The most stringent method to identify selection pressure at protein level is to compare the rate of synonymous substitution (d*S*) and non synonymous substitution (d*N*). The nucleotide sequences coding the extracellular domains were aligned with ClustalW. Comparisons between orhtologous TLR genes of chicken and turkey were performed. Positive (d*S* <d*N*) or purifying (d*S* >d*N*) selection was tested with codon based z-test using the Nei-Gojobori method (P-distance) at 5% significance level.

3.6.3. Expression analysis of TLRs in duck and turkey tissues by real-time PCR

3.6.3.1. Experimental birds

Day-old ducklings and turkey poults (n=6) (*Meleagris gallapova*) were sacrificed humanely and tissue samples were collected aseptically from various tissues such as heart, liver, intestine, bursa, bone marrow (BM), muscle and spleen and immediately frozen before further analysis. Each frozen tissue samples were homogenized and total RNA extracted using Trizol as described in section 3.2.1.1 and reverse transcribed into cDNA as per section 3.2.1.2.

3.6.3.2. Quantitative analysis of TLRs mRNA in duck and turkey tissues by real-time PCR

Relative quantification of TLRs (*TLR1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15* and *21*) in duck and turkey tissues was done by real-time PCR using the Mx3000P QPCR system (Stratagene, USA) as described earlier in section 3.2.1.4. Primers were designed from published/available duck and turkey TLR sequences and annotated TLR sequences using online IDT website (eu.idtdna.com/Scitools/Applications/RealTime PCR) following default options. The primer sequences designed for duck and turkey TLRS and their respective amplicon size are given in Table 3.4 and Table 3.5 respectively.

3.6.3.3. Statistical analysis

Relative gene expression of TLRs expressed as 40-ΔCt mean values were analyzed by one-way ANOVA with Tukey's post-hoc test using SPSS software (version 10.0). Values were considered significant at $P<0.01$.

3.7. Molecular characterization of TLR15 of avian species

3.7.1. Amplification and sequencing of TLR15

To amplify the full length ORF of Aseel, Kadaknath, Japanese quail and turkey *TLR15* overlapping primer sets were designed based on published broiler chicken *TLR15* mRNA sequence (DQ267901; Higgs *et al.*, 2006) and annotated turkey *TLR15* sequence (ENSMGAG00000015891). The primer sets used were designed to amplify the fragments exactly covering full length ORF from cDNA samples and are presented in Table 3.6. cDNA was prepared from spleen tissue of Aseel, Kadaknath, Japanese quail and turkey as described in section 3.2.1.1 and 3.2.1.2. The reaction conditions for amplification in PCR were same for all the fragments. The 50 µl PCR reaction contained 50 pmol of each forward and reverse primers, 1 µl template cDNA, 200 µM of dNTP mix, 1.0 mM $MgCl_2$ and 2.5 U Taq DNA polymerase (MBI Fermentas, USA) in 1X Taq buffer. Amplification conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. PCR amplicons verified by 1% agarose gel electrophoresis were purified and sequenced by using an automated DNA sequencer (ABI prism, model 377, version 3.0).

3.7.2. Sequence analysis of avian *TLR15*

The sequences of the fragments were aligned using MegAlign of DNA star software (Lasergene, USA) and complete coding sequences was identified and translated to amino

Table 3.4. Duck TLR genes specific real-time primer sequences used in the present study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Product size	Accession no./Ensembl no.
TLR1LA	GCGTTAACTTTGGAGCGTGTTCGT	CGGACAAAGCATGTGTGGCATGTA	182	FJ477859.1
TLR1LB	TTTCCAAGGTGAGCTCCATGACCA	ACTCTGTCAGCTGGTTTGAGGACA	153	FJ477859.1
TLR2A	TGGTACATGAGAATGACGTGGGCA	TTTCCACCCAGTTGGAGTCGTTCT	124	FJ477862.1
TLR2B	TCCTAACTTGGTGGCCCTGTCAAT	TATCCCTGCCTGGTGCTGAATGAA	160	FJ477862.1
TLR3	TCAGTACATTTGCAACACTCCGCC	AAATGGAGCACTGTCTTTGCAGGC	88	ENSAPLG0000008976
TLR4	ATCGGCTCTCCTTCACTTCATGCT	TTCGTGTGCTGGAGGTCCAAGTAT	151	ENSAPLG00000012625
TLR5	AGCTGGCTCACAGTTTGACAGGTA	ATCTGAGTGCCTGCTGGAGTTCAT	113	ENSAPLG0000001279
TLR7	GTCGCCTCACAGAAGTCCCCAGAG	CCAACCTGACAGGCACGCAGTTGC	156	DQ888644
TLR15	CATCACAGCTTGTGTTGCCGTTCT	TGCCATACACCAGTACCAACCCAT	106	ENSAPLG0000009929
TLR21	ACGACAGCTTCGTGTCCTACAACT	ACGATGTTGTCGATGATGCTGC	142	ENSAPLG00000014726
ß actin	CCGTAAGGACCTGTACGCCAACAC	GCTGATCCACATCTGCTGGAAGG	208	AY251275

Table 3.5. Turkey TLR genes specific real-time PCR primer sequences used in the present study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Product size	Accession no./Ensembl no.
TLR1LA	TGTGCATCTACCTGGATGTGCTGT	AACGAATCGCGCTCTCTGTACGAT	148	FJ477857
TLR1LB	TGTGCATCTACCTGGATGTGCTGT	ATGAAGGCGTGAAACTGCAGAACG	124	FJ477858
TLR2A	AGAACGACTCCAACTGGGTGGAAA	AGAGCGTCTTGTGGCTCTTCTCAA	156	FJ477860
TLR2B	AGAACGACTCCAACTGGGTGGAAA	AGAGCGTCTTGTGGCTCTTCTCAA	156	FJ477861
TLR3	ACCCGGATTGCAGTCTCAGTACAT	AAATGGAGCGCTATCTTTGCAGGC	103	ENSMGAG00000011425
TLR4	AGACGCCTCCGCATCTTGGATATT	TGTAAGGGCTTGGAGTGGCTTGTA	155	ENSMGAG0000005422
TLR5	TGAACTCCAGCAGACACTCAGGTT	TGCTGGTGGATGGCTTCCTATCAA	160	ENSMGAG00000015929
TLR7	TGATGCAGTGTGGTTTGTTGGGTG	AACCAAGCTCCTTCCTTTGTGTGC	112	ENSMGAG00000014706
TLR15	AGCGTCCAACTGCTCCATTGTAGA	GCATGGAAATCCGATTGCTGCTGA	98	ENSMGAG00000015891
TLR21	ACAGCTGCACAACATTTCCTTCCG	TGTAAAGGTCGGTCAGCAGGTTGT	194	ENSMGAG00000015581
ß actin	AGACATCAGGGTGTGATGGTTGGT	TGGTGACAATGCCGTGTTCAATGG	118	ENSMGAG00000003960

acid sequence. The signal peptide of the sequence was identified by SignalP program (www.cbs.dtu.dk/service/SinalP). The domain structure, LRRs and transmembrane region were identified by SMART (Schultz *et al.,* 1998; http://www.smartembl-heidelberg.de/) and TMHMM (Letunic *et al.,* 2004; http://www.cbs.dtu.dk/service/TMHMM/) respectively.

3.8. Functional characterization of duck TLRs

3.8.1. *In vitro* stimulation of PBMC culture with TLR agonists

Pooled blood samples with heparin, collected from six day-old ducklings were used for the study. The peripheral blood mononuclear cells (PBMC) were separated by using Histopaque-1.007. The cells were washed twice in RPMI-1640 medium (Sigma Chemical Co., USA) and resuspended in fresh RPMI-1640 and the cell concentration was adjusted to 1×10^7 cells/ml. Then these cells were dispensed into a 6 well cell culture plate (2 ml/well) and incubated at 37^{o}C. The PBMCs were stimulated with TLR agonists, i.e. lipopolysaccharide (LPS) and poly I: C with a dose of 20 and 50 µg /ml, respectively and incubated at 37^{o}C in a 5% CO_2 and 95% humidified atmosphere. Treated cells were harvested at 12 and 24 hr intervals with 1 ml of Trizol in DEPC treated 1.5 ml eppendorf tubes and further processed for RNA isolation.

3.8.2. Quantitative analysis of cytokine mRNAs in duck PBMC after *in vitro* stimulation by real-time PCR

Expression levels of cytokine *IL-1ß* gene in LPS stimulated duck PBMC culture and IFN-? in poly I:C stimulated culture and both genes in unstimulated control PBMC culture were studied by real-time PCR using specific primers as mentioned in Table. Total RNA was extracted from cells harvested at 12 and 24 h in Trizol and first strand cDNA was synthesized as described earlier in section 3.2.1.1. and 3.2.1.2 respectively. Relative levels of cytokine gene expressions in samples were quantified by real-time PCR using the Mx3000P QPCR system (Stratagene, USA) as described in section 3.2.1.4. Primer sequences are given in Table 3.7.

Ct values were normalized with housekeeping gene ß-actin values. Values are calibrated with unstimulated control and fold-changes in gene expressions were calculated as $2^{-\Delta\Delta Ct}$(Livak

Table 3.6. Primer sequences used to amplify full length coding region of avian *TLR15* gene

Target gene	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amplicon size (bp)**	**Annealing position in coding region (bp)**
1	ATGAGGATCCTTATTGGGAG	GCTGTCAGCTCTTCATTAGA	660	1-659
2	TGACTTGTGTGGAGCACCGAT	TGGAGCAGTTGGACACTT	608	589-1192
3	TACACCCATCGAAAGCCT	GATGGCGTTGTCGCTAATGT	598	1134-1731
4	ATCAGGGAATAAGATCTC	TACAGTTCATACTGACACCA	653	1652-2345
5	GGAAACTGATGGATTCAAGATA	TCATTCCATCTCAATTACATCC	421	2187-2607

Table 3.7. Duck cytokine genes specific primer sequences used in the present study

Gene name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	Product size	Accession no./ Ensembl no.
IFN?	CAAGTAATTCGGATGTAGC	GCGTTGGATTTTCAAGCC	153	AF087134
IL-1ß	ACCAGAAGTGCTTCGTGCTTGAGT	GCGCAATGTTGAGCCTCACTTTCT	96	DQ393268
ß actin	CCGTAAGGACCTGTACGCCAACAC	GCTGATCCACATCTGCTGGAAGG	208	AY251275

and Schmittgen, 2001). Statistical comparisons of fold changes in gene expression between stimulated and unstimulated samples were performed using General Linear Model in version 9.2 of SAS software with a P value <0.05 considered significant.

3.9. Cloning and prokaryotic expression of chicken *AvBD2*

3.9.1. Isolation of genomic DNA from blood

Genomic DNA was isolated from the venous blood samples from White Leghorn chicken by using phenol-chloroform extraction method as described by Sambrook and Russell (2001).

3.9.2. Amplification, cloning and sequence confirmation of chicken *AvBD2* gene

The entire mature peptide region (40 amino acids) coded by exon 3 (119 bp) of *AvBD2* gene was amplified by standard procedure using gene specific primers designed to include *Nde* I/ *Xho* I restriction sites in forward and reverse primers respectively (Table 3.8). Chicken *AvBD2* gene sequence organization and strategy followed were described in Fig 3.1. Primer sequences were designed based on the published chicken *AvBD2* gene sequence available in NCBI database (accession no. AF033336). The composition of PCR reaction was as follows:

Table 3.8. Primer sequences used to amplify chicken mature peptide region of *AvBD2* gene

Sequence	Primer sequence (5'-3')	Accession no.
Forward*	CAGTGA **CATATG**TTGTCTTCGCCCCG	AF033336
Reverse**	TCACTG**CTCGAG**CCATTTGCAGCAGG	

Sequence in bold include *Nde I and **Xho I restriction enzyme digestion site.

Template DNA	1 µl
Forward primer	400 ng
Reverse primer	400 ng
dNTPs (2.5 mM each)	4 µl
10 X PCR buffer	10 µl

Taq DNA polymerase (3 U/ µl)	1 µl
Water to make the final volume to	100 µl

PCR was carried out in a thermocycler (Eppendorf, Germany) using the following conditions: One cycle of initial denaturation was carried out at 94°C for 5 min followed by thirty five cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec), extension (72°C for 2 min) and final extension at 72°C for 5 min. The PCR product was purified and was cloned in pUC29 cloning vector after *Nde*I / *Xho*I enzyme digestion following standard procedure. Positive clones after transformation in DH5a cells were selected by colony PCR. The presence of insert was checked by digestion of positive plasmids with *EcoRI* and *Hind III* restriction enzymes (sites present on the vector backbone outside the insert location) digestion. Two positive clones were sequenced in either direction using vector specific primers in automated gene sequencer (ABI prism, model 377, version 3.0). The nucleotide sequence was aligned with AF033336 to confirm the sequence identity.

3.9.3. Expression of recombinant chicken ß-defensin AvBD2 in *E. coli* system

The coding region (143 bp including restriction enzyme region) of chicken AvBD2 mature peptide was sub-cloned into Nde I/ Xho I restriction sites in pET-28a expression vector (Novagen, WI) adopting standard cloning procedures. The recombinant plasmid (pET-28a-Gga_AvBD2) DNA was transformed into competent BL21 (DE3) (Promega, WI) *E. coli* cells and transformants were grown on Luria Bertani (LB) plates containing 50 µg/ml Kanamycin. Single colony from the plate was inoculated into LB broth and incubated at 37°C until the OD_{600} reached 0.5.

For checking the production of recombinant chicken AvBD2 protein, log phase cultures were induced at 37°C by adding IPTG (isopropyl-ß-D-thiogalactoside; Promega) to a final concentration of 1 mM. Aliquots collected at 0 to 5 h post induction were analyzed on a 12% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

3.9.4. Purification of recombinant chicken AvBD-2

Recombinant chicken *AvBD2* was purified from 100 ml of induced cultures under denaturing conditions by nickel-nitrilotriacetic (Ni NTA) acid resin affinity chromatography by

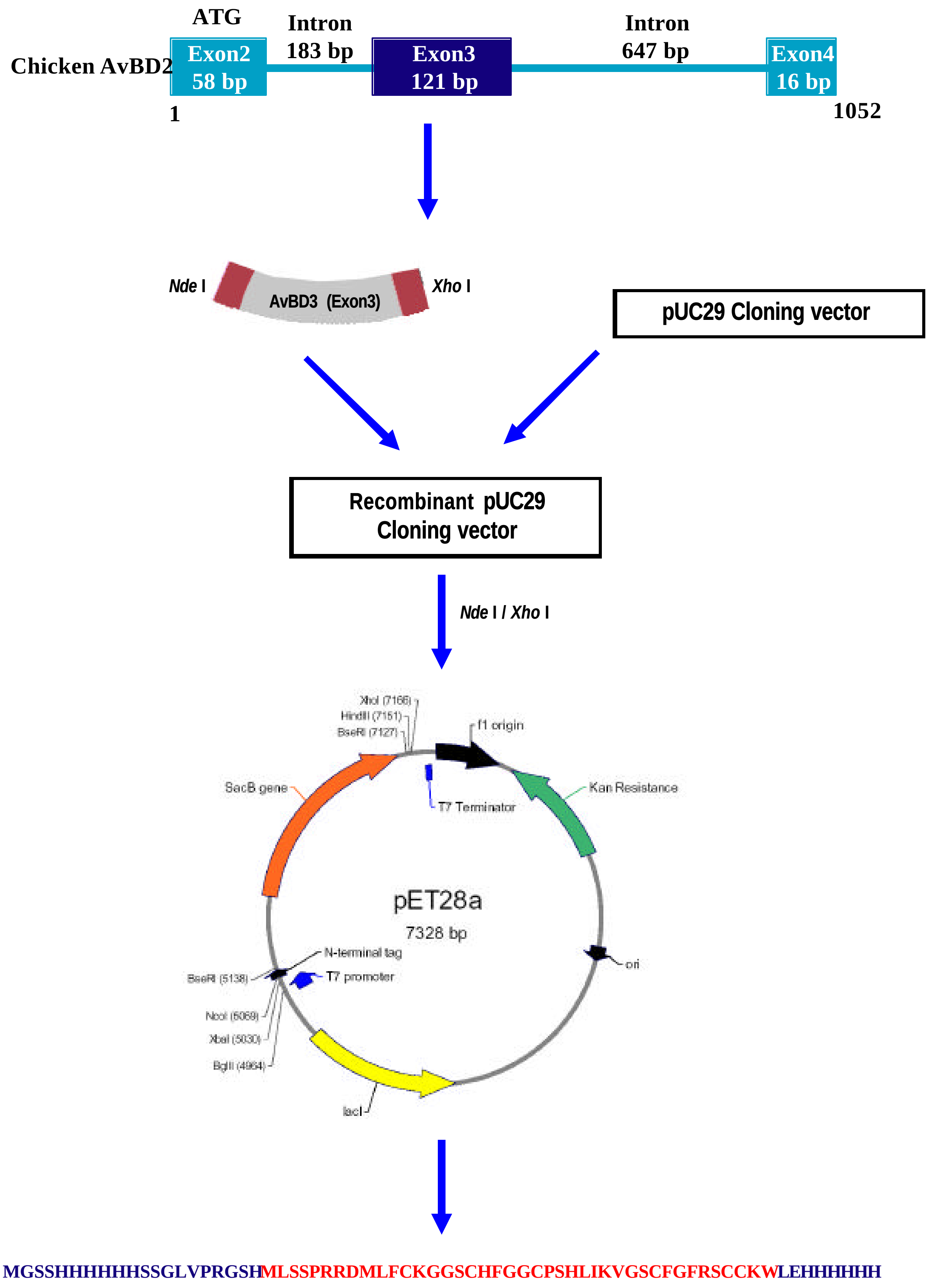


Fig. 3.1 : Strategy followed for amplification, cloning and expression of chicken AvBD2

using Qiagen kit following manufacturer's instructions. Briefly, the bacterial cells pelleted by centrifugation at 1800 x g were resuspended in 5 ml of sonication buffer (buffer A; 8 M urea, 10 mM NaH_2PO_4 and 10 mM Tris; pH-8.0). The cell suspension was sonicated at 15 hz for one minute each for 3 times with one min interval. The sonicated cell suspension was centrifuged at 10,000 x g for 30 min to remove cell debris. To the clear supernatant 500 µl of 50% Ni NTA slurry was added and shaked at 200 rpm for 1 h. The supernatant with slurry was loaded into the column and washed with four bed volume of urea lysis buffer (buffer B). The column was again washed with 20 bed volume of wash buffer (buffer C). The bound protein was eluted 8 times with 250 µl elution buffer (buffer E). The first four tubes of elutes were pooled and dialysed against large volume of PBS containing 0.1% Triton X100 at 4°C. The eluted recombinant protein was checked in SDS-PAGE, quantified by Bradford assay and stored at -80°C until further use.

3.9.5. Evaluation of antimicrobial activity of recombinant chicken AvBD2

Minimum Bactricidal Concentration (MBC) of recombinant chicken AvBD2 was determined by microtitre broth dilution method. Virulent isolate of *Salmonella enterica* serovar Pullorum obtained from the National Salmonella Center, Indian Veterinary Research Institute, India was used. Mid-log-phase cultures of the test organisms were diluted in LB broth to reach a density of 10^6 CFU/ml. 10 µl of this diluted culture was treated with 250 µl recombinant chicken AvBD2 (5-50 µg/ml in PBS; with 136 mM NaCl and 2.68 mM KCl) in a 96-well microtiter plate for 3 h at 37°C. LB broth (100 µl) was added after 3 h and plates were further incubated for 12 h. The bacterial growth was detected by measuring the absorbance at 570 nm. The growth inhibition was further confirmed by plating the contents of the wells, showing no visible growth of bacteria, onto LB agar plates and incubating at 37°C for 12-18 h. The MBC was calculated as the lowest concentration of the recombinant chicken AvBD2 that prevents any residual colony formation.

* * *

4

Results

4.1. Standardization of molecular techniques used in this study

4.1.1. RNA isolation, cDNA preparation

Total RNA was isolated from samples and concentration of the RNA was estimated spectrophotometrically and was found to be ~500 ng of total RNA isolated from 50mg of tissue samples. Spectrophotometric reading at OD_{260} and OD_{280} represented the purity of the total RNA and the ratio of $OD_{260/280}$ ranged between 1.9 and 2.0 indicating purity and lack of DNA and protein contamination. The cDNA was checked by PCR amplification of ß-actin gene. A 184 bp products confirmed the amplification of the ß-actin gene.

4.1.2. Primer effficiency analysis

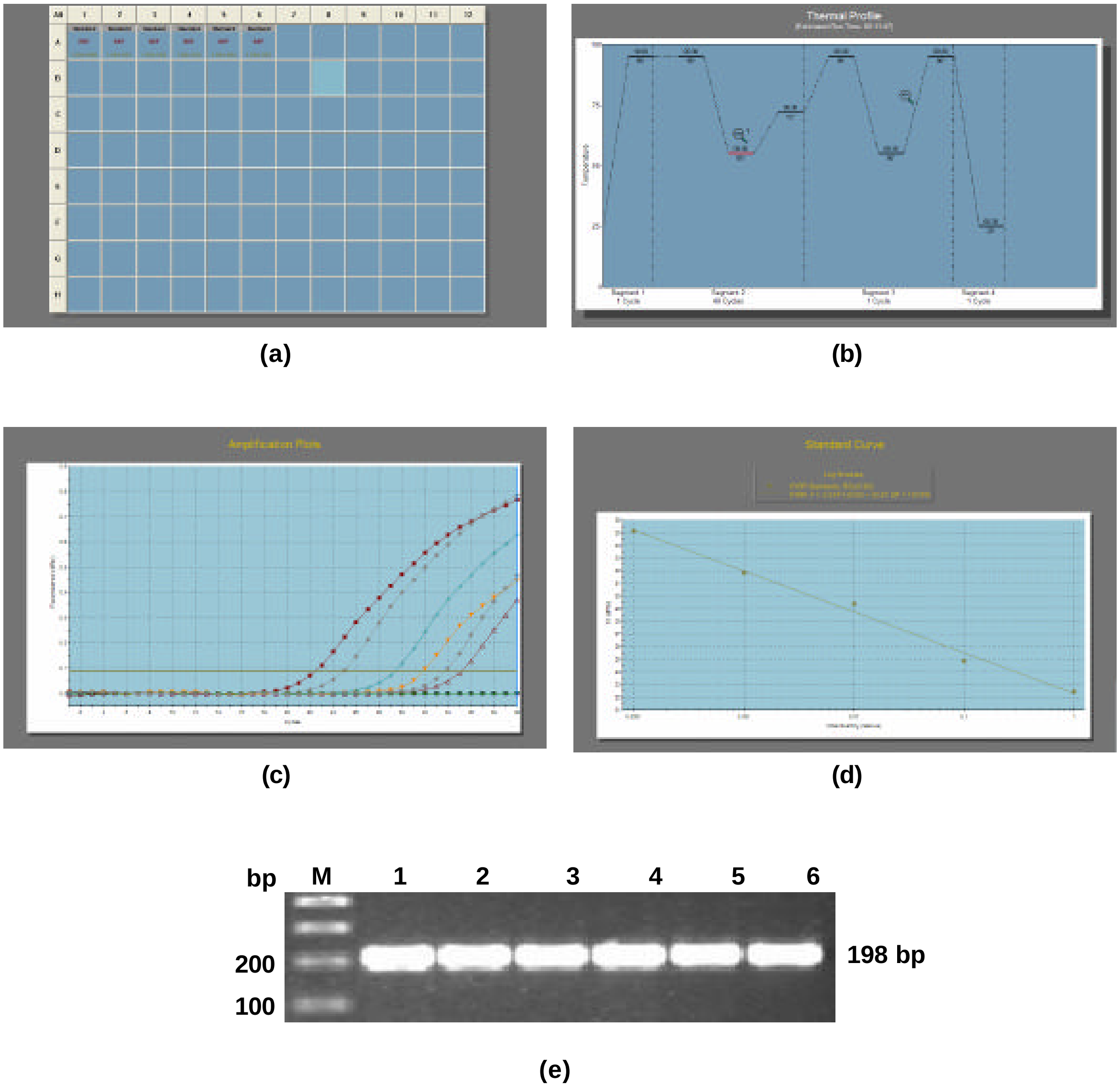
Standard curves for each primer pair were generated using serially diluted transcribed RNA sample (Fig. 4.1). Regression analysis of the Ct (threshold cycle) values of standard curve was done to calculate slope and amplification efficiency (Fig. 4.1d). Efficiencies of the primer pairs used in the study ranged between 93-100%.

4.1.3. Real-time PCR analysis

Ct values were generated at the end of each real-time PCR assay. Ct values ranged from ~15-30. The specificity of the amplified products was confirmed from Tm^{o}C (temperature melting) value from dissociation curve analysis (Fig 4.2). No template control (NTC) included in each real-time PCR batch failed to amplify any specific products which confirmed the absence of any contamination of PCR master mix. Non-reverse transcribed RNA of each sample used

Fig. 4.1 : **Representative primer efficiency analysis by standard curve in real-time PCR assay**

(a) Plate setup using serially diluted cDNA sample
(b) Thermal profile setup
(c) Amplification plot
(d) Standard curve indicating slope and amplification efficiency
(e) Representative agarose gel electrophoresis of amplified PCR product (*TLR3*)



(a)

(b)

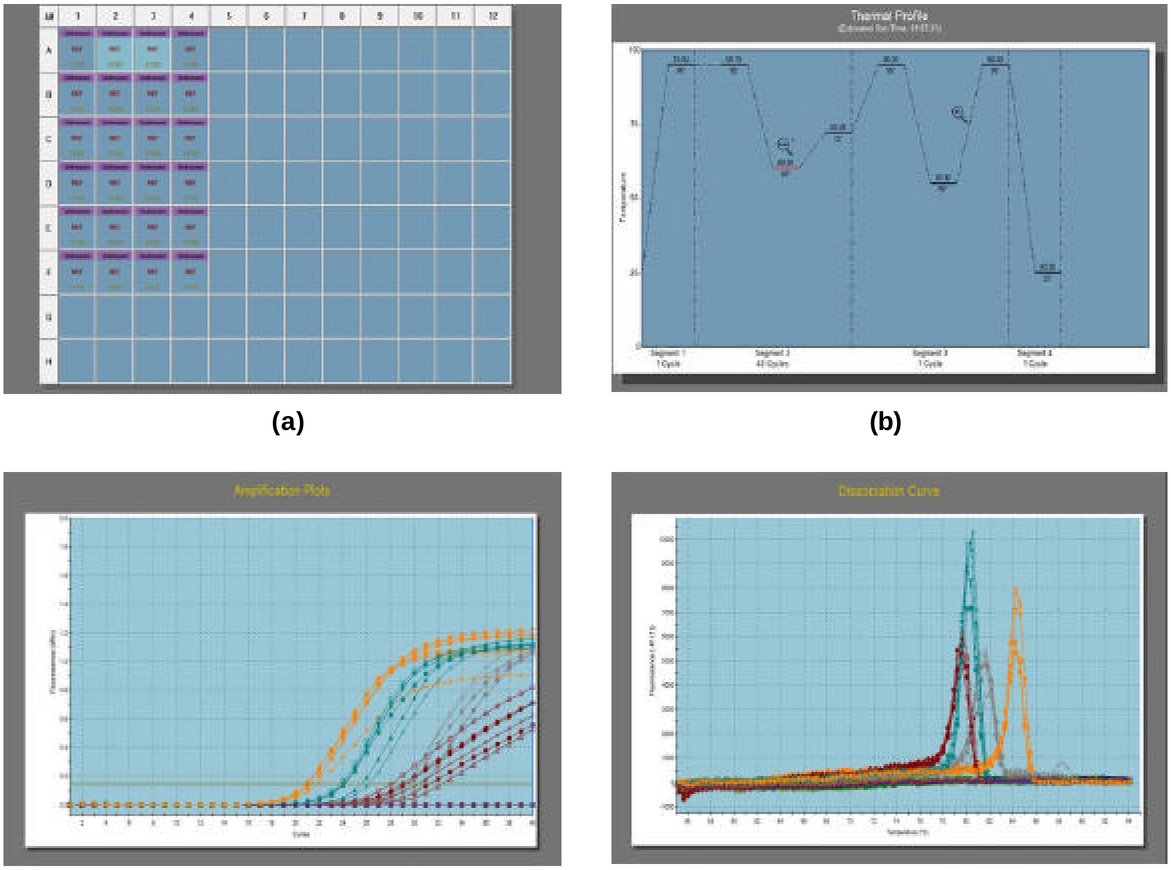
(c)

(d)

(e)

Fig. 4.2 : **Representative quantitative real-time PCR analysis for gene expression study**

(a) Plate setup
(b) Thermal profile setup
(c) Amplification plot
(d) Dissociation curve indicating melting curve for four different genes



(a) (b)

(c) (d)

in real-time PCR assays failed to amplify. This confirmed the absence of contamination of samples with genomic DNA.

4.2. Comparative quantification of constitutive *TLR*s mRNA expression in Indian native and White Leghorn chickens

Measurable expression was observed for all six *TLR*s (*3, 4, 5, 7, 15* and *21*) in all seven tissues (heart, liver, spleen, intestine, bursa, bone marrow (BM) and muscle) of both Indian native breeds and White Leghorn. The results are presented in Table 4.1 There was no significant difference in *TLR3* gene expression among the breeds (P=0.75), however among the tissue type there was significant difference (P=0.01) (Table 4.1a).The interaction effect of tissue type and breed on *TLR3* gene expression was not significant (P=0.55). Among the tissue type lymphoid organs spleen and bursa and non-lymphoid organ heart, expressed significantly higher *TLR3* gene followed by intestine, BM, liver and muscle. Significant (P=0.001) difference was found among breeds in *TLR4* gene expression, however there was no significant difference among tissue type (P=0.12) and interaction effect between tissue type and breed on the gene expression (P=0.07). Aseel and White Leghorn expressed higher levels of *TLR4* gene than Kadaknath in different tissues investigated (Table 4.1b). For rest of the TLR gene expressions (*TLR5, 7, 15* and *21*), the interaction effect between tissue type and breed was significant (P<0.01). *TLR5* gene expression was significantly (P<0.01) higher in liver, spleen and intestine of Aseel and bone marrow of Kadaknath than White Leghorn tissues (Table 4.1c). *TLR7* gene expression was significantly (P<0.01) higher in liver of White Leghorn than Aseel and Kadaknath tissues (Table 4.1d). *TLR15* gene expression was significantly (P<0.01) higher in spleen of Aseel than Kadaknath and White Leghorn tissues (Table 4.1e). *TLR21* gene expression was significantly (P<0.01) higher in bursa and heart of Aseel in comparison to Kadaknath White Leghorn tissues (Table 4.1f).

4.3. Differential *TLR*s mRNA expression patterns during chicken embryological development

All seven investigated *TLR*s (*2A, 3, 4, 5, 7, 15* and *21*) genes were expressed in whole embryonic tissue as early as 3rd embryonic day (ED) (Fig. 4.3). Measurable expression

Table 4.1. Constitutive *TLR*s mRNA expression levels in tissues of Aseel, Kadaknath and White Leghorn chicken (a) TLR3; (b) TLR4; (c) TLR5; (d) TLR7; (e) TLR15; (f) TLR21

Tissue	Aseel	Kadaknath	White Leghorn
(a) TLR3			
Heart	38.90±0.35^{a}	38.24±0.86^{a}	37.65±2.13^{a}
Liver	37.49±0.27^{ab}	36.38±0.76^{ab}	39.63±0.84^{ab}
Spleen	38.50±0.43^{a}	38.02±0.19^{a}	37.97±0.07^{a}
Intestine	37.58±0.05^{ab}	38.62±1.37^{ab}	37.84±0.38^{ab}
Bursa	38.51±1.31^{a}	39.64±0.24^{a}	37.53±0.48^{a}
Bone marrow	35.69±0.69^{ab}	37.17±0.53^{ab}	38.46±0.31^{ab}
Muscle	36.00±0.05^{b}	36.17±1.91^{b}	35.44±0.32^{b}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values with different superscripts between rows differ significantly (P<0.01).

Table 4.1. Contd...

Tissue	Aseel	Kadaknath	White Leghorn
(b) TLR4			
Heart	37.59±0.11^{a}	34.69±2.75^{b}	39.29±0.04^{a}
Liver	39.10±0.02^{a}	35.41±1.91^{b}	38.11±1.47^{a}
Spleen	37.82±0.02^{a}	39.78±0.15^{b}	39.58±0.28^{a}
Intestine	39.78±0.14^{a}	37.64±1.70^{b}	38.42±0.32^{a}
Bursa	38.29±0.86^{a}	38.71±0.11^{b}	38.95±0.10^{a}
Bone marrow	37.25±0.19^{a}	36.82±0.60^{b}	39.28±0.02^{a}
Muscle	38.42±0.17^{a}	34.56±0.16^{b}	38.92±0.60^{a}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values with different superscripts between rows differ significantly (P<0.01).

Table 4.1. Contd...

Tissue	Aseel	Kadaknath	White Leghorn
(c) TLR5			
Heart	35.00 ± 0.09^{defg}	37.94 ± 0.25^{ab}	36.24 ± 0.22^{bcdef}
Liver	39.41 ± 0.12^{a}	38.28 ± 0.53^{ab}	36.77 ± 0.02^{bcde}
Spleen	39.36 ± 0.33^{a}	36.84 ± 0.48^{bcd}	31.96 ± 0.12^{i}
Intestine	39.56 ± 0.08^{a}	37.45 ± 0.74^{abc}	32.36 ± 0.81^{hi}
Bursa	36.19 ± 0.19^{bcdef}	37.91 ± 0.11^{ab}	35.56 ± 0.30^{cdef}
Bone marrow	33.02 ± 0.44^{ghi}	39.22 ± 0.01^{a}	36.49 ± 0.54^{bcde}
Muscle	34.12 ± 0.14^{fgh}	39.33 ± 0.44^{a}	34.69 ± 0.31^{efg}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values with different superscripts differ significantly (P<0.01).

Table 4.1. Contd...

Tissue	Aseel	Kadaknath	White Leghorn
(d) TLR7			
Heart	37.15 ± 0.04^{abcde}	36.0 ± 0.39^{abcdef}	37.17 ± 2.27^{abcde}
Liver	34.93 ± 0.11^{def}	36.61 ± 0.14^{abcdef}	39.77 ± 0.20^{a}
Spleen	39.13 ± 0.61^{abc}	37.94 ± 0.70^{abcd}	39.39 ± 0.08^{ab}
Intestine	36.51 ± 0.19^{abcdef}	36.13 ± 0.24^{abcdef}	39.51 ± 0.03^{ab}
Bursa	35.94 ± 0.23^{abcdef}	35.73 ± 0.12^{abcdef}	36.57 ± 0.36^{abcdef}
Bone marrow	34.49 ± 0.23^{def}	33.83 ± 0.48^{ef}	37.23 ± 0.02^{abcde}
Muscle	37.77 ± 1.69^{abcd}	32.72 ± 0.31^{f}	35.30 ± 0.16^{cdef}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values with different superscripts differ significantly (P<0.01).

Table 4.1. Contd...

Tissue	Aseel	Kadaknath	White Leghorn
(d) TLR15			
Heart	37.65±0.34^{abcde}	39.80±0.14^{ab}	38.11±0.80^{abcde}
Liver	36.23±0.09^{def}	39.52±0.17^{abc}	37.28±0.33^{cde}
Spleen	39.94±0.01^{a}	38.81±0.43^{abc}	37.75±1.06^{abcde}
Intestine	37.65±0.66^{abcde}	39.37±0.37^{abc}	37.48±0.17^{abcde}
Bursa	37.22±0.44^{cde}	39.31±0.09^{abc}	38.73±0.04^{abc}
Bone marrow	34.80±0.55^{f}	38.48±0.09^{abcd}	38.41±0.02^{abcd}
Muscle	35.98±0.30^{ef}	37.52±0.46^{bcde}	39.08±0.08^{abc}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values with different superscripts differ significantly (P<0.01).

Table 4.1. Contd...

Tissue	Aseel	Kadaknath	White Leghorn
(d) TLR21			
Heart	38.23±0.22^{abcde}	26.94±0.45^{c}	37.07±0.54^{abcde}
Liver	39.23±0.13^{abc}	27.48±0.37^{fg}	37.77±0.05^{abcde}
Spleen	35.70±0.39^{e}	28.21±0.79^{fg}	36.60±1.34^{bcde}
Intestine	39.45±0.35^{ab}	26.56±0.15^{g}	37.18±0.18^{abcde}
Bursa	39.83±0.01^{a}	26.83±0.15^{g}	35.69±0.31^{e}
Bone marrow	38.60±0.12^{abcd}	29.90±1.07^{f}	36.49±0.15^{cde}
Muscle	39.65±0.07^{a}	25.47±0.64^{g}	36.05±0.08^{de}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values with different superscripts differ significantly (P<0.01).

were found for all seven TLRs (*2A, 3, 4, 5, 7, 15* and *21*) in all tissues (heart, liver, intestine and brain) investigated during embryo development. The fold changes in *TLR* gene expression among different tissues during different embryonic days (EDs) were significant ($P<0.01$) (Table 4.2). The kinetics of *TLR* gene expressions is presented in fig. 4.4. Four of the seven TLRs gene expressions were significantly ($P<0.01$) higher (*TLR2*; 2.85 fold, *TLR3*; 3.57 fold; *TLR4*; 46.52 fold and *TLR7*; 2.94 fold) at 12 ED relative to expression at day 3 ED, whereas *TLR15* gene expression was significantly ($P<0.01$) higher on 7 ED (0.64 fold) and *TLR5* (2.16 fold) (Fig. 4.4g) and *TLR21* (6.45 fold) were highly expressed on ED 18. Relatively low level of differential expression of *TLR2A* was observed in embryonic liver and intestine whereas declining trend of *TLR2* expression was found in brain and heart tissue during development (Fig. 4.4a). Among all the TLRs investigated *TLR4* was the highest expressed gene (Fig. 4.4c) and *TLR15* was the lowest in all tissues during chicken embryo development (Fig. 4.4f). Tissue wise analysis of gene expression of TLRs showed that liver expressed significantly ($P<0.01$) higher levels of most of the genes (*TLR2*; 2.75 fold, *TLR4*; 32.72 fold and *TLR21*; 5.44 fold) followed by intestine in which *TLR2* (2.7 fold), *TLR3* (2.3 fold) and *TLR7* (2.5 fold), whereas least change in *TLR* gene expressions was observed in brain, *TLR5* gene expression was significantly higher ($P<0.01$) in brain tissue (1.5 fold) (Fig. 4.4d). However no significant difference was found in *TLR15* gene expression among the tissues during development. Relatively low level of differential expression for *TLR2A* and *TLR15* was observed in intestine and liver whereas declining trend of gene expression was observed in heart and brain during embryonic development. Most substantial change in expression was observed for *TLR4* (in liver on12th ED). Significant ($P<0.01$) interaction effect between tissue and ED was also observed.

4.4. Comparative quantification of constitutive ß-defensins mRNA expression in Indian native and White Leghorn chickens

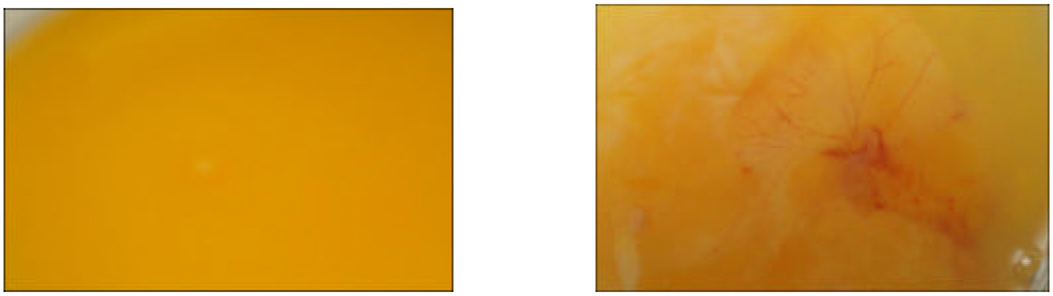
Measurable *AvBD* (*1-14*) mRNA expression was observed in the spleen of all three breeds. However, only *AvBD5*, *12* and *14* genes were differentially expressed among these breeds and no significant difference was found for rest of the *AvBD*s (Table 4.3). Differential expression of *AvBD* (*1-14*) mRNA in spleen of Aseel, Kadaknath and White Leghorn as mean 40-^{o}Ct±SEM and fold difference in gene expression in native chickens in comparison to

Fig. 4.3 : ***TLR* mRNA expression in developing chicken embryo**

(a, b & c) Agarose gel electrophoresis of amplified TLR gene products

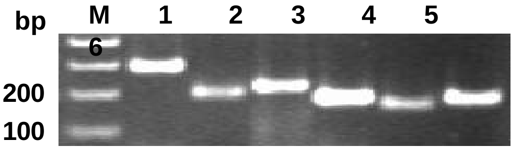
Lane M : 100 bp DNA ladder

Lane 1 : TLR2A; **Lane 2 :** TLR3; **Lane 3 :** TLR4 : **Lane 4 :** TLR5; **Lane 5 :** TLR7; **Lane 6 :** TLR15

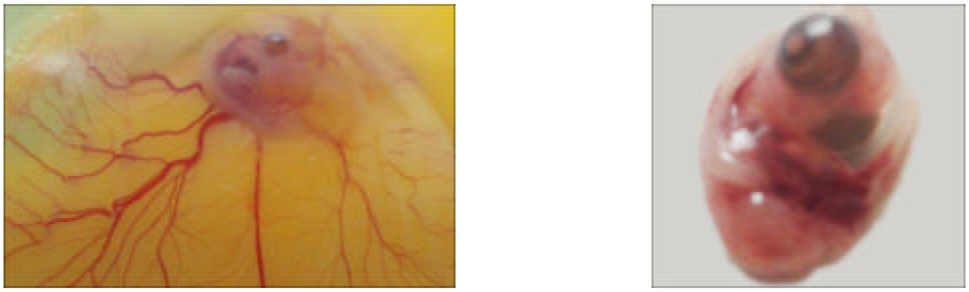


(A) Fertilized egg showing germinal disc

(B) 3 day embryo

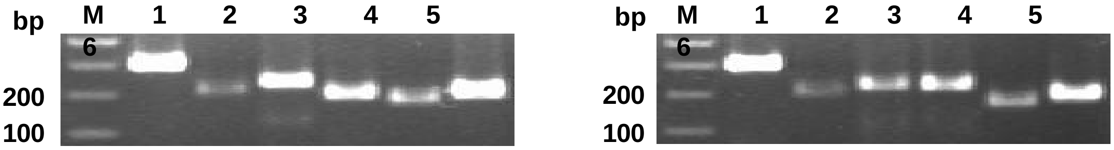


(a)



(C) 5 day embryo

(D) 7 day embryo



(b)

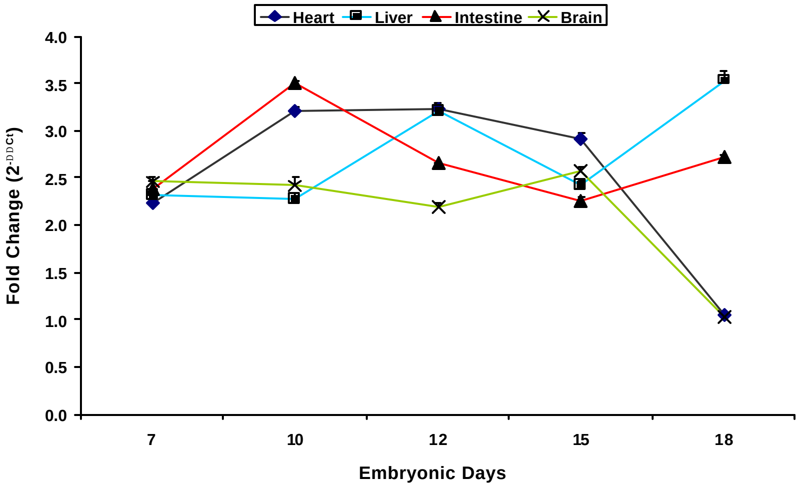
(c)

Table 4.2. Fold changes in *TLR* gene expressions during chicken embryo development

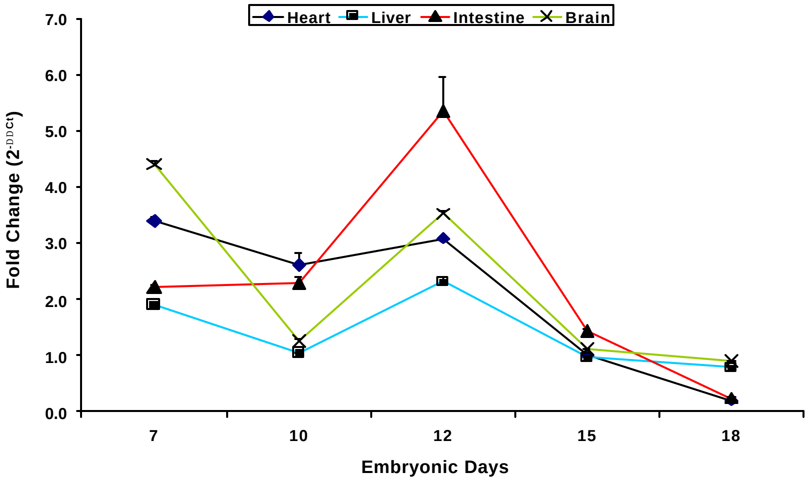
Gene	5ED	7ED	9ED	12ED	15ED	18ED
TLR2A	Heart	2.24^{g}	3.21^{b}	3.23^{b}	2.91^{c}	1.05^{h}
	Liver	2.32^{fg}	2.27^{g}	3.21^{b}	2.43^{efg}	3.54^{a}
	Intestine	2.38^{efg}	3.50^{a}	2.65^{cde}	2.26^{g}	2.72^{cd}
	Brain	2.45^{defg}	2.42^{efg}	2.19^{g}	2.57^{def}	1.03^{h}
TLR3	Heart	3.40^{c}	2.61^{de}	3.08^{cd}	1.0^{g}	0.20^{i}
	Liver	1.89^{ef}	1.05^{g}	2.31^{e}	0.96^{gh}	0.78^{ghi}
	Intestine	2.20^{e}	2.30^{e}	5.36^{a}	1.44^{fg}	0.24^{hi}
	Brain	4.40^{b}	1.26^{fg}	3.52^{c}	1.11^{g}	0.89^{ghi}
TLR4	Heart	21.38^{bcd}	2.30^{f}	8.98^{def}	18.29^{cde}	6.55^{ef}
	Liver	23.49^{bc}	0.69^{f}	82.68^{a}	34.12^{b}	22.63^{bcd}
	Intestine	9.15^{def}	0.87^{f}	20.96^{bcd}	21.68^{bcd}	0.64^{f}
	Brain	27.21^{bc}	0.78^{f}	73.45^{cde}	18.50^{cde}	5.14^{ef}
TLR5	Heart	1.76^{cd}	1.18^{efg}	0.63^{hi}	1.32^{de}	1.1^{efg}
	Liver	0.73^{ghi}	0.60^{hi}	0.55^{i}	2.19^{bc}	2.19^{bc}
	Intestine	1.25^{ef}	0.87^{fghi}	0.81^{fghi}	2.60^{ab}	2.60^{ab}
	Brain	2.63^{ab}	0.63^{hi}	0.61^{hi}	2.75^{a}	2.75^{a}
TLR7	Heart	2.85^{bc}	2.43^{bcd}	3.03^{b}	1.03^{h}	2.19^{cde}
	Liver	1.90^{def}	1.03^{h}	1.94^{def}	1.03^{h}	2.40^{bcd}
	Intestine	1.56^{efgh}	2.45^{bcd}	5.69^{a}	1.48^{efgh}	1.63^{efgh}
	Brain	2.19^{cde}	1.33^{fgh}	1.11^{gh}	1.83^{defg}	1.83^{defg}
TLR15	Heart	0.86^{a}	0.66^{abcd}	0.23^{cde}	0.17^{de}	0.21^{de}
	Liver	0.38^{abcde}	0.28^{bcde}	0.44^{abcde}	0.24^{cde}	0.37^{abcde}
	Intestine	0.60^{abcd}	0.49^{abcde}	0.62^{abcd}	0.24^{bcde}	0.56^{abcde}
	Brain	0.79^{abc}	0.38^{abcde}	0.84^{ab}	0.03^{e}	0.22^{de}
TLR21	Heart	5.62^{c}	2.32^{def}	1.37^{efgh}	0.45^{h}	1.86^{defg}
	Liver	2.59^{de}	1.54^{defgh}	1.23^{fgh}	10.0^{b}	11.85^{a}
	Intestine	2.58^{de}	1.83^{defg}	1.12^{fgh}	2.77^{d}	11.39^{a}
	Brain	1.35^{efgh}	0.79^{gh}	1.55^{defgh}	4.40^{c}	0.71^{gh}

Values are mean $2^{-\Delta\Delta Ct}$ values using 3ED (Embryonic day) as calibrator. Values with different superscript differ significantly ($P<0.01$) in each TLR.

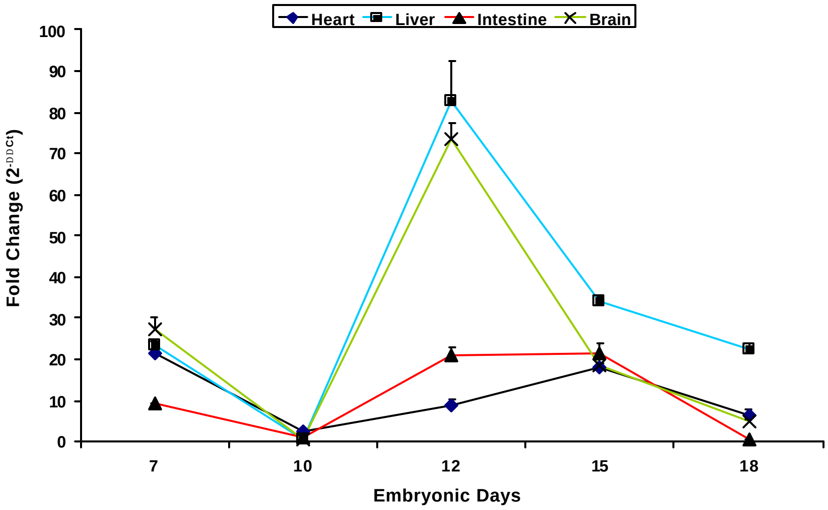
Fig. 4.4 : The kinetics of *TLRs* gene expressions in chicken embryonic tissues during development
(a) TLR2A; **(b)** TLR 3; **(c)** TLR4; **(d)** TLR5; **(e)** TLR7; **(f)** TLR15; **(g)** TLR21
Values are mean $2^{-\Delta\Delta}$Ct values using 3 ED as calibrator



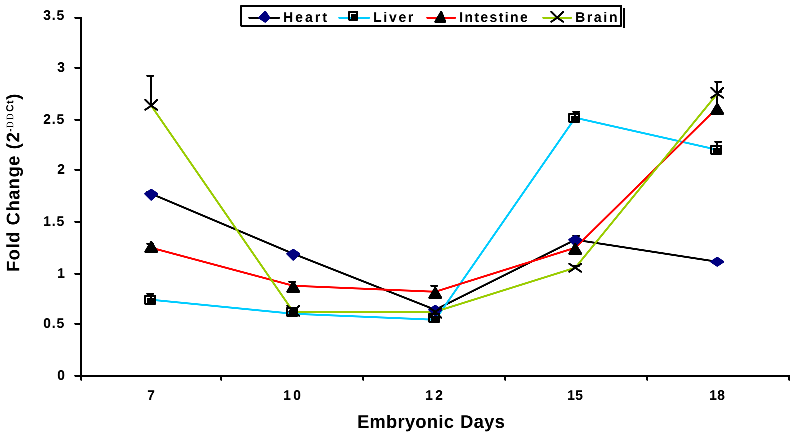
(a) TLR2A



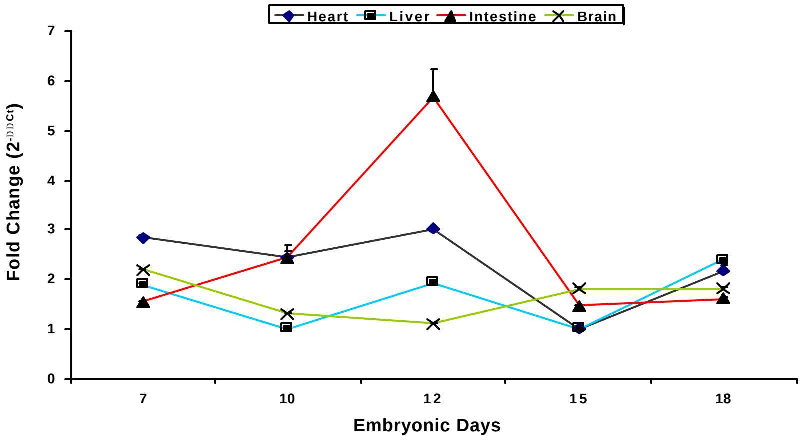
(b) TLR3



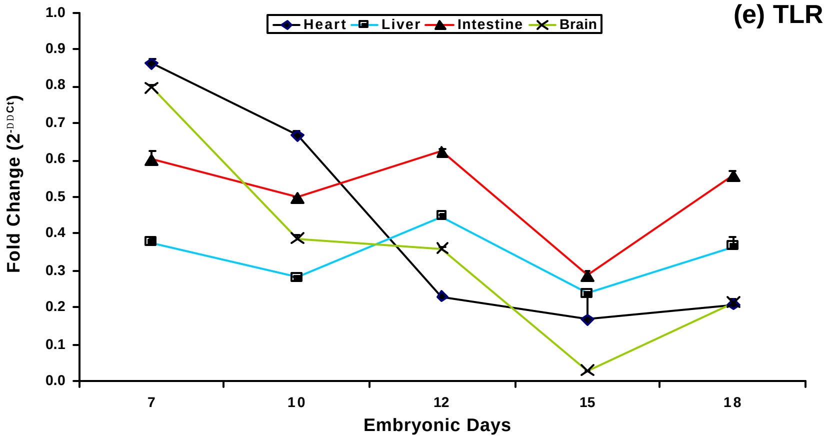
(c) TLR4



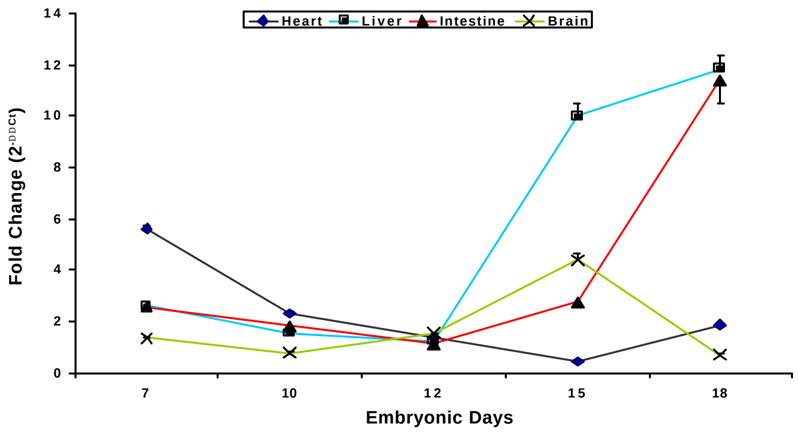
(d) TLR5



(e) TLR7



(f) TLR15



(g) TLR21

Table 4.3. Differential expression of *AvBD (1-14)* mRNA in spleen of Aseel, Kadaknath and White Leghorn chicken

Gene	Aseel		Kadaknath		White Leghorn
	Mean±SEM	FD	Mean±SEM	FD	Mean±SEM
AvBD1	38.88±0.91	1.65	39.4±0.06	2.17	37.23±1.25
AvBD2	29.54±3.21	-0.72	30.32±0.75	0.06	30.26±1.97
AvBD3	31.89±2.43	1.84	30.02±0.25	-0.03	30.04±1.67
AvBD4	31.90±4.12	1.93	37.31±0.83	7.34	29.97±1.15
AvBD5	**35.83±1.89**^{ab}	4.35	**38.76±0.57**^{a}	7.28	**31.48±1.82**^{b}
AvBD6	35.92±3.07	-1.89	38.63±0.60	0.82	37.81±1.04
AvBD7	30.66±0.95	4.76	28.6±2.01	2.69	25.9±0.17
AvBD8	35.18±2.58	0.02	32.63±1.51	-2.52	35.16±0.67
AvBD9	33.07±2.47	-1.43	32.22±0.98	-2.29	34.51±0.43
AvBD10	35.36±1.76	4.29	32.09±0.94	1.03	31.07±0.32
AvBD11	34.40±1.32	-1.25	33.27±0.45	-2.38	35.65±0.36
AvBD12	**36.60±2.66**^{a}	8.58	**24.42±1.11**^{c}	-3.61	**28.03±0.28**^{b}
AvBD13	33.60±2.24	-1.76	33.60±0.66	-1.75	35.35±1.16
AvBD14	**37.28±1.01**^{a}	1.51	**32.42±0.35**^{b}	-3.3	**35.77±0.19**^{ab}

Values are expressed as mean 40-ΔCt±SEM (n=6 for each breed). Values in bold with different superscripts are significantly different ($P<0.01$). FD: fold difference in gene expression in comparison to White Leghorn. Positive values indicate the higher gene expression and negative represents lower gene expression in native breeds in comparison to White Leghorn.

White Leghorn are presented in Table 4.3. *AvBD5* gene expression was significantly (P=0.043) higher in Kadaknath than Aseel and White Leghorn. *AvBD12* was differentially expressed among these three breeds and was significantly (P=0.005) higher in Aseel in comparison to Kadaknath and White Leghorn. *AvBD14* gene expression was significantly (P=0.04) higher in Aseel than other two breeds in the present investigation. Among the *AvBD* genes investigated in chicken spleen, *AvBD7* gene was the most expressed and *AvBD1* gene was the least expressed in spleen of both Aseel and White Leghorn chicken (Significant at P<0.01). However, *AvBD5* was the highest expressed and *AvBD12* was the least expressed in Kadaknath (Significant at P<0.01).

4.5. Differential innate immune gene expressions in the gastrointestinal tract of chicken upon *S*. Pullorum-infection

4.5.1. *TLR*s gene expression in the gastrointestinal tract (GI) of *Salmonella* serovar Pullorum-infected broiler chicken

Measurable TLRs (*1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15* and *21*) mRNA expression was observed in both infected and uninfected GI tract tissues of 3-day-old broiler chickens. TLR gene expression in control and infected chicken GI tissues are presented in Fig. 4.5. The results of fold-changes in TLR gene expressions in infected group as calculated by $2^{-\Delta\Delta Ct}$ method are presented in Table 4.4.

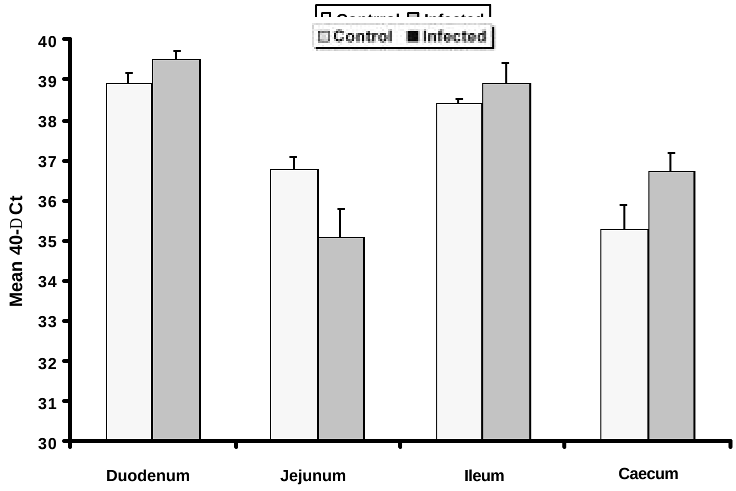
In GI segments with significant differences all had upregulated *TLR* (*TLR2, TLR4*, *TLR21*) expression after *S*. Pullorum infection. The exceptions were for *TLR3*, with one fold (P=0.009) and for *TLR15*, with 0.8 fold (P=0.04) decrease in the expression levels in jejunum after infection. *TLR4* gene expressions were significantly upregulated in duodenum (0.5 fold; P=0.04) and ileum (1.6 fold; P=0.001). Gene expressions for some of the TLRs (*TLR1LA*, *ILB*, *2B* and *TLR5*) remained unchanged after infection with *S*. Pullorum in all GI tissues studied. Ileum of infected chicken had changes in the expression levels in the greatest number of TLRs, including *TLR2*, *TLR4* and *TLR21*. The least changes in *TLR*s gene expression were observed in caecum of infected chicken wherein increased expression of *TLR21* (1.73 fold; P=0.01) was observed while other TLRs expressions remained unchanged. Most substantial changes in gene expression were found for *TLR21*, being upregulated in all the investigated GI tissues (D: 3.7 fold; P=0.001, J: 2.16 fold; P=0.03, I: 1.9 fold; P=0.007; C: 1.7 fold; P=0.01) 24 h post challenge with *S*. Pullorum.

Table 4.4. Fold-changes in *TLR* mRNA expression in gastrointestinal tissues of 3-day-old broiler chickens infected with *Salmonella enterica* serovar Pullorum

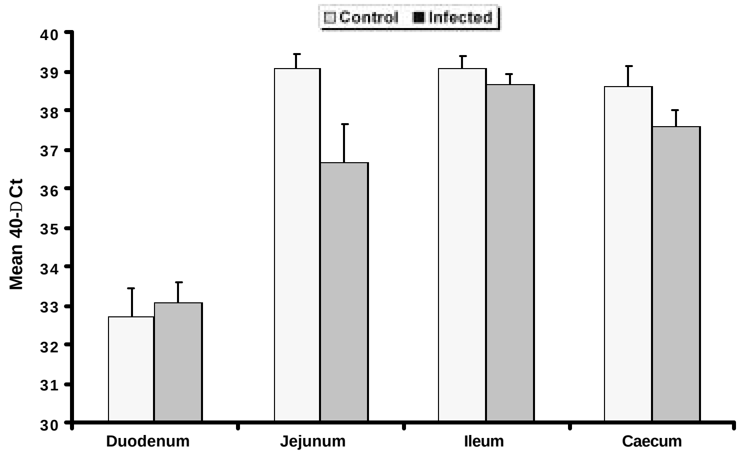
Gene	Duodenum	Jejunum	Ileum	Caecum
TLR1LA	0.59	-1.69	0.47	1.43
TLR1LB	0.36	-2.42	-0.41	-1.04
TLR2A	0.60	0.53	**1.17****	0.53
TLR2B	0.58	0.58	0.49	0.30
TLR3	-0.74	**-1.29****	-0.34	-1.09
TLR4	**0.54***	0.39	**1.61****	-0.38
TLR5	0.21	-1.94	-0.22	0.08
TLR7	0.27	-1.69	-1.57	-0.73
TLR15	0.76	**-0.89***	-0.36	0.32
TLR21	**3.70****	**2.16****	**1.90****	**1.73***

Data are expressed as $2^{\Delta\Delta Ct}$. Positive values indicate infected chicken have an increase in respective TLR gene expression compared to uninfected control in respective tissue. *Significantly different at P<0.05; ** Significantly different at P<0.01. Statistical analysis was performed on individual 40-ΔCt values.

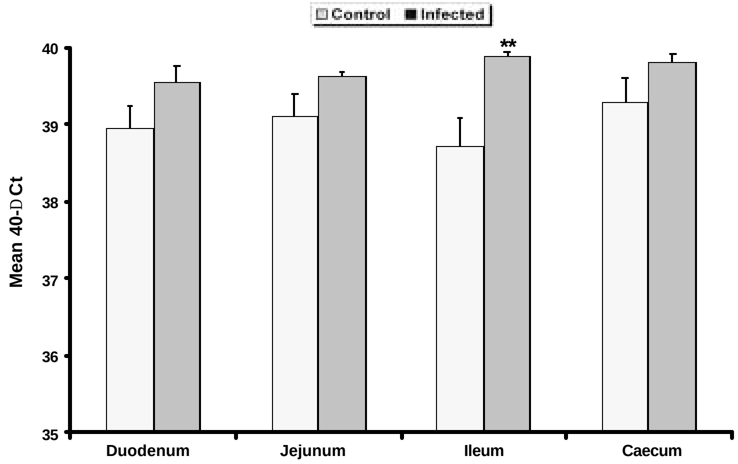
Fig. 4.5 : ***TLR*s gene expressions in the gastrointestinal tissues of *S.* Pullorum infected broiler chicken**
(a) *TLR1LA*; **(b)** *TLR1LB*; **(c)** *TLR2A* ; **(d)** *TLR2B*; **(e)** *TLR3*; **(f)** *TLR4*; **(g)** *TLR5*; **(h)** *TLR7*; **(i)** *TLR15*; **(j)** *TLR 21*
Values are mean 40-ΔCt±SEM (n = 6 for each group)
*$P \leq 0.05$; **$P \leq 0.01$



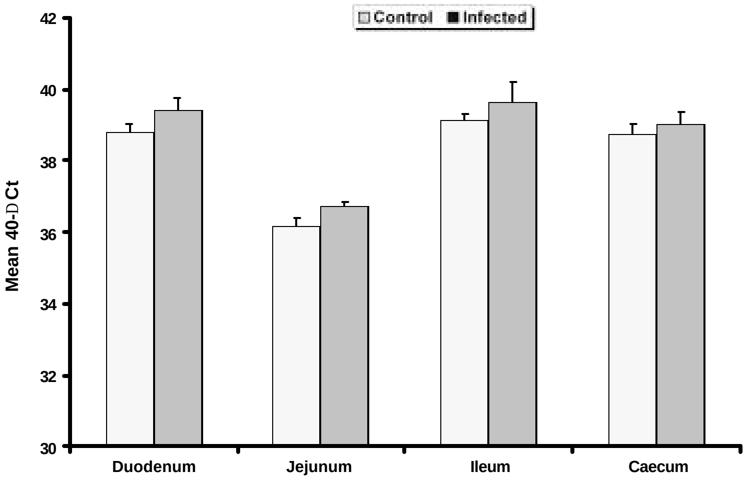
(a) TLR1LA



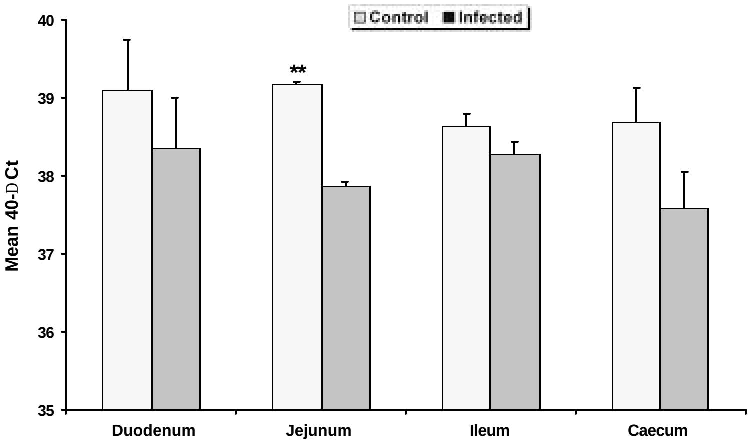
(b) TLR1LB



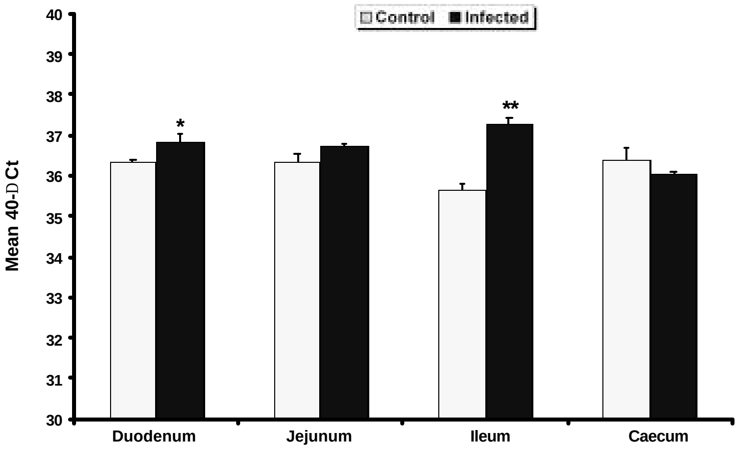
(c) TLR2A



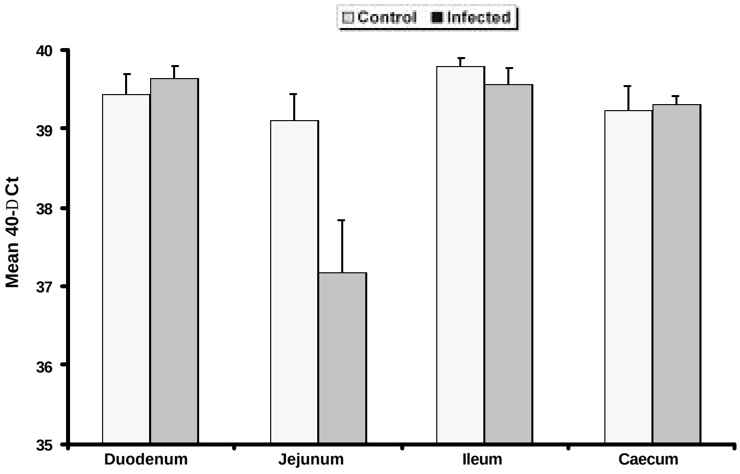
(d) TLR2B



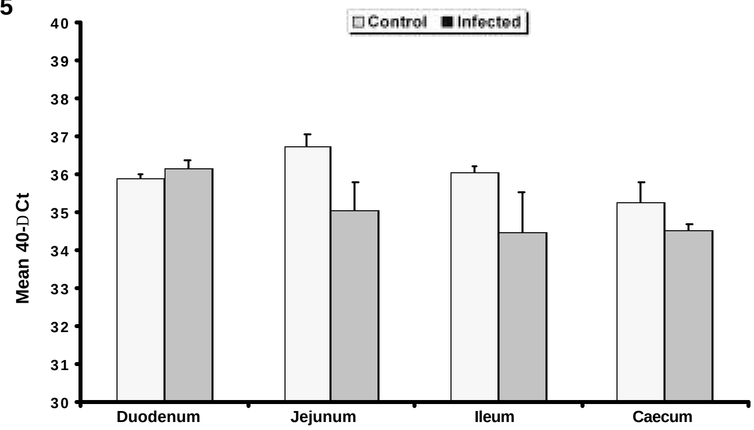
(e) TLR3



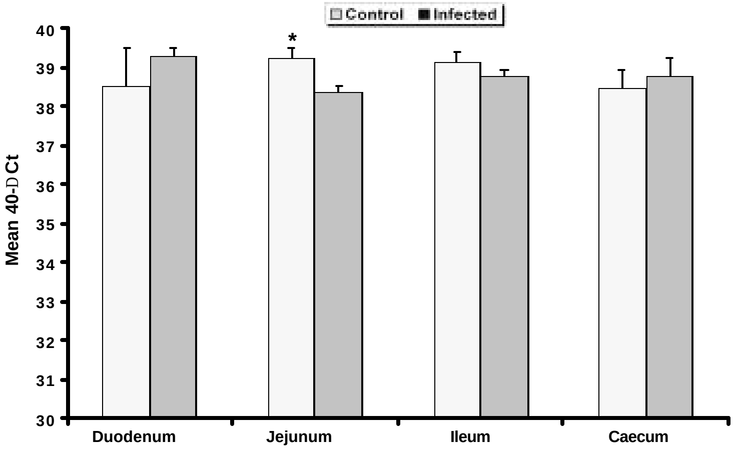
(f) TLR4



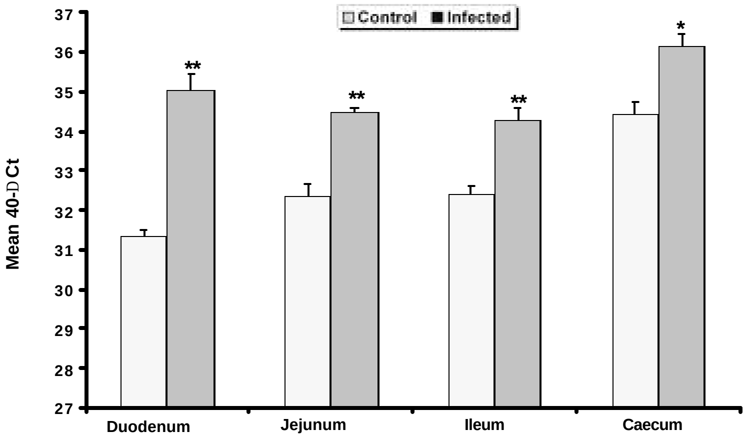
(g) TLR5



(h) TLR7



(i) TLR15



(j) TLR21

4.5.2. ß-Defensins gene expression in the gastrointestinal tract of *Salmonella* serovar Pullorum-infected broiler chicken

Expression analysis revealed that out of 14 *AvBD* genes, all were detectable in all GI tissues in both uninfected and infected groups, while expressions of *AvBD2* and *7* were at very low level in the both groups (Table 4.5). In the present study, quantitative real-time PCR analysis revealed significant upregulation of *AvBD3* in caecum (4.3 fold; P=0.02), *AvBD4* in duodenum (5.5 fold; P=0.005), *AvBD5* in duodenum (3.1 fold; P=0.04), jejunum (3.19 fold; *P*=0.007) and ileum (3.38 fold; P=0.01), *AvBD6* in duodenum (0.87 fold; P=0.01) and *AvBD12* in duodenum (4.76 fold; P=0.01) and jejunum (2.91 fold; P=0.003) respectively. Significant down regulation in the expressions of *AvBD10* in caecum (2.04 fold; P=0.02), *AvBD11* in duodenum (1.72 fold; P=0.002) and ileum (3.93 fold; P=0.02), *AvBD13* in ileum (1.95 fold; P=0.02) and *AvBD14* in ileum (2.46 fold; P=0.014) and caecum (2.09 fold; P=0.03), respectively, were observed. Whereas 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 changes in gene expression were found for *AvBD5*, being significantly (P<0.05) upregulated in most of the GI tissues investigated (Fig. 4.6).

4.6. Identification, molecular characterization and expression analysis of *TLR* genes of avian species

4.6.1 *In silico* identification of duck and turkey *TLR* genes

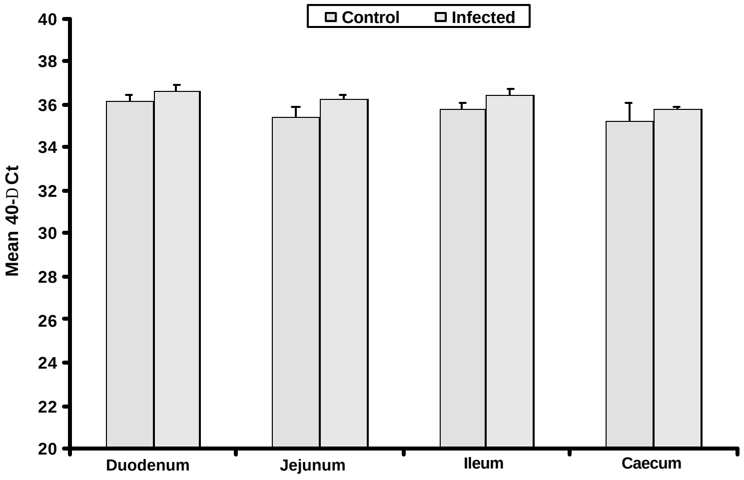
Attempts were made to identify *TLR* genes of duck and turkey from NCBI and genome database of duck and turkey (Ensembl turkey genome browser) using chicken and zebra finch TLRs as query sequences (Table 3.3). We identified TLR genes (*TLR1LA, 1LB, 2A*, *2B, 3, 4, 5, 7, 15* and *21*) orthologs of chicken TLR repertoires in both duck (Table 4.6) and turkey (Table 4.7) genome. NCBI database search and *in silico* analysis revealed that both duck and turkey genome possess 10 *TLR* genes orhtologous to chicken TLR repertoire. To identify the status of *TLR8*, *9* and *10* in duck and turkey genome, corresponding human sequences were used for blast search. Similar to chicken no orthologs of these genes could be found in both duck and turkey genome.

Table 4.5. Fold-changes in *AvBD* (*1-14*) mRNA expression in gastrointestinal tissues of 3-day-old broiler chickens infected with *Salmonella enterica* serovar Pullorum

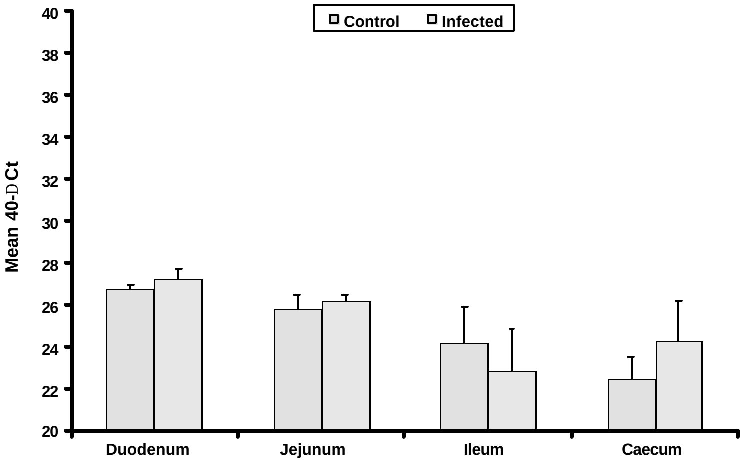
Gene	Duodenum	Jejunum	Ileum	Caecum
AvBD1	0.53	0.87	0.69	0.54
AvBD2	0.47	0.33	-1.37	1.87
AvBD3	1.9	1.39	**-1.73***	**4.33****
AvBD4	**5.50***	-0.01	-1.10	-0.39
AvBD5	**3.10***	**3.19****	**3.38***	-0.09
AvBD6	**0.87***	1.70	**-1.53****	-0.18
AvBD7	0.99	-0.05	-0.63	0.41
AvBD8	0.17	-0.12	-0.31	0.77
AvBD9	0.72	**0.30***	-1.2	-0.49
AvBD10	-0.52	0.73	1.48	**-2.04***
AvBD11	**-1.72****	0.01	**-3.93****	-0.70
AvBD12	**4.76***	**2.91****	0.53	-3.58
AvBD13	0.46	1.45	**-1.95***	0.54
AvBD14	0.62	0.74	**-2.46***	**-2.09***

Data are expressed as $2^{-\Delta\Delta Ct}$. Positive values indicate infected chicken have an increase in respective AvBD gene expression compared to uninfected control in respective tissue. *Significantly different at P<0.05; ** Significantly different at P<0.01. Statistical analysis was performed on individual 40-ΔCt values.

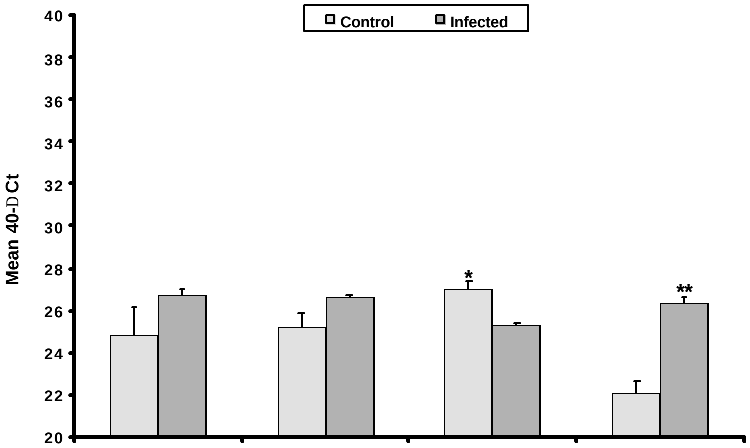
Fig. 4.6 : ***AvBD* gene expressions in the gastrointestinal tissues of *S.* Pullorum infected broiler chicken (a)** *AvBD1*; **(b)** *AvBD2*; **(c)** *AvBD3*; **(d)** *AvBD4*; **(e)** *AvBD5*; **(f)** *AvBD6*; **(g)** *AvBD7*; **(h)** *AvBD8*; **(i)** *AvBD9*; **(j)** *AvBD10*; **(k)** *AvBD11*; **(l)** *AvBD12*; **(m)** *AvBD13*; **(n)** *AvBD14*
Values are mean 40-ΔCt±SEM (n = 6 for each group)
$*P \leq 0.05$; $**P \leq 0.01$



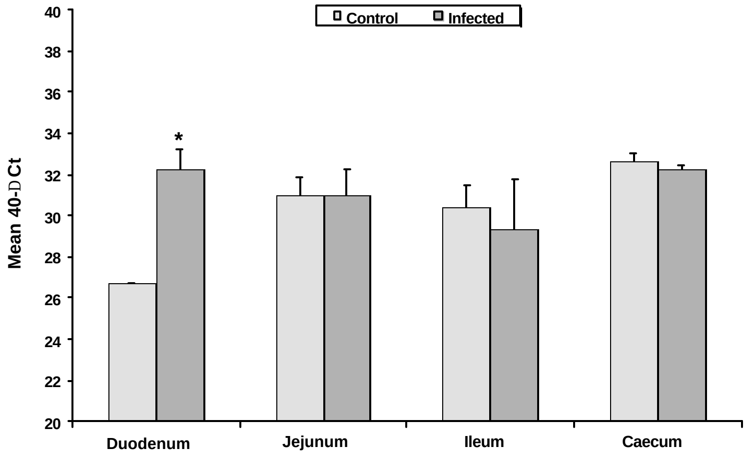
(a) AvBD1



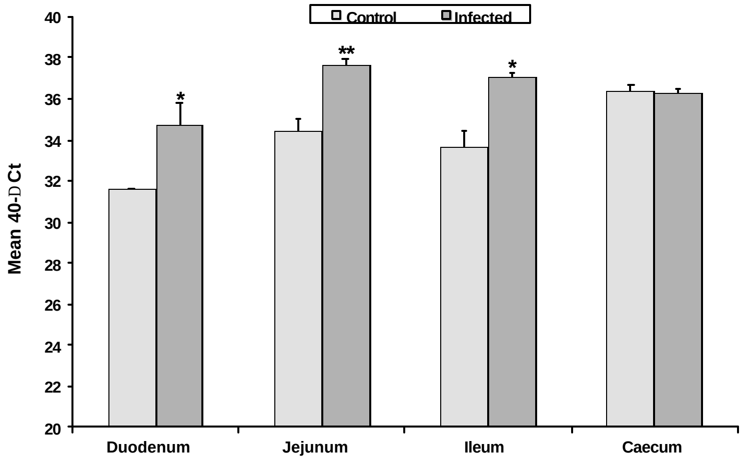
(b) AvBD2



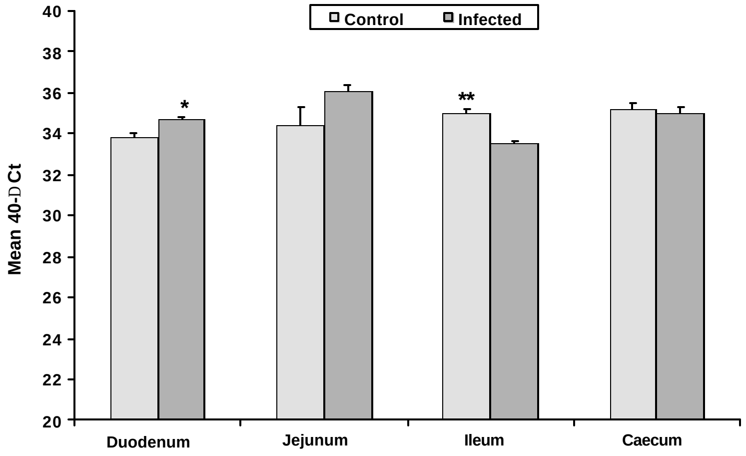
(c) AvBD3



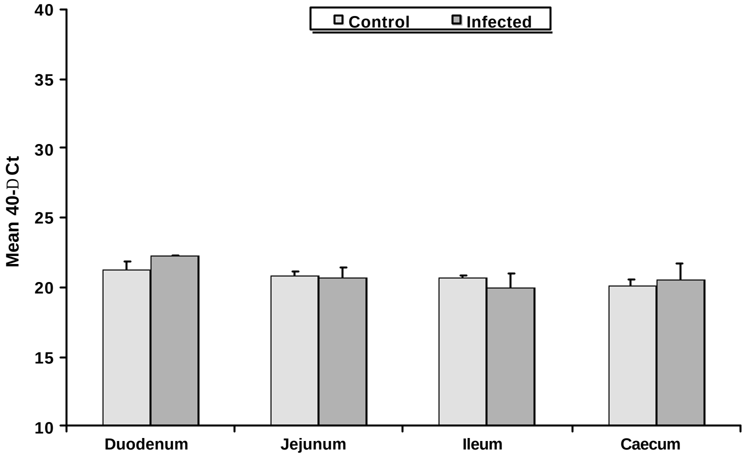
(d) AvBD4



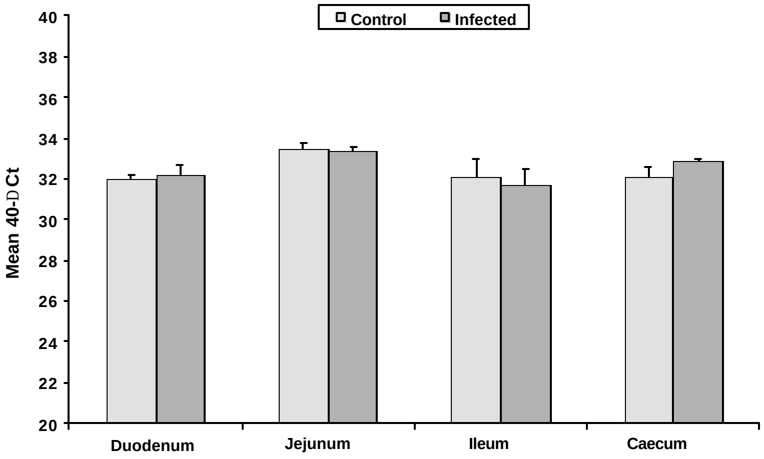
(e) AvBD5



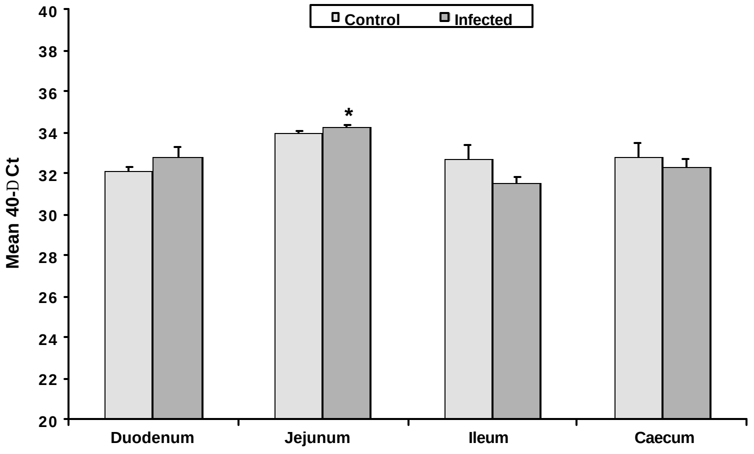
(f) AvBD6



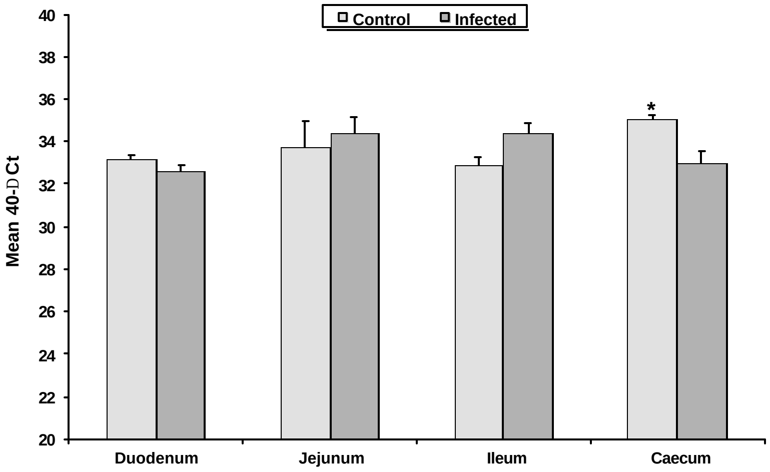
(g) AvBD7



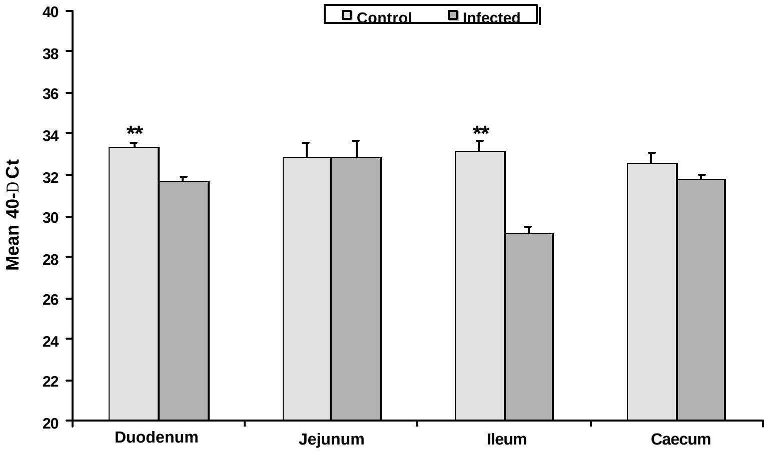
(h) AvBD8



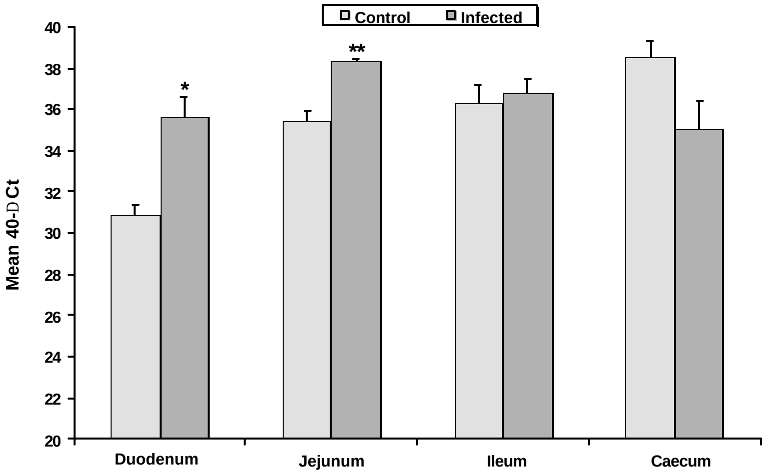
(i) AvBD9



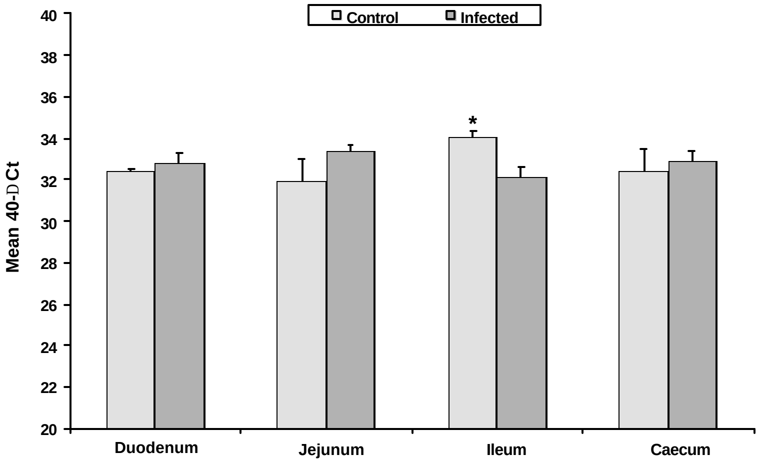
(j) AvBD10



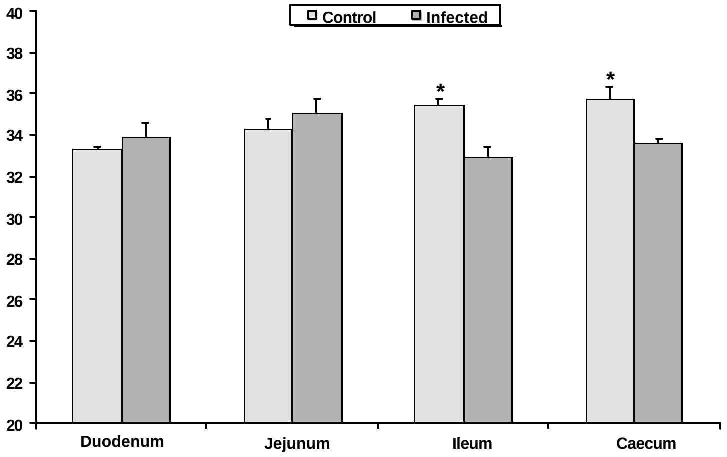
(k) AvBD11



(l) AvBD12



(m) AvBD13



(n) AvBD14

Table 4.6. TLR gene repertoire in duck (*Anas platyrynhos*) genome

Gene name	Accession no/ Gene ID	Location Scaffold	Exons transcript	Length of (bp)	Length (aa)	% id[a]
TLR1A	FJ477859.1	43095-45551	1	2456	818	81.8
TLR1B	FJ477859.1	17445-19397	1	1952	650	80.1
TLR2A	FJ477862.1	14425-16806	1	2373	793	81.9
TLR2B	FJ477862.1	22976-25327	1	2352	783	81.9
TLR3	ENSAPLG0000008976	19:217675-223928	4	3204	899	83.1
TLR4	ENSAPLG00000012625	1381:116840-122803	5	2538	846	81.4
TLR5	ENSAPLG0000001279	421:310497-313067	1	2571	857	80.4
TLR7	DQ888645	2282:21806-20090	2	3147	1048	84.4
TLR15	ENSAPLG0000009929	629:2572220-591289	3	2526	842	82.5
TLR21	ENSAPLG00000014726	7715:2813-4757	5	1749	583	77.5

The accession numbers of NCBI or gene ID of pre-ensemble duck genome draft are listed. [a]% id- Percentage identity of duck TLR genes with corresponding chicken orthologs at amino acid level.

Table 4.7. TLR gene repertoire in turkey (*Meleagris gallapova*) genome

Gene name	Accession no/ Gene ID	Chromosome Location	Exons transcript	Length of (bp)	Length (aa)	% id[a]
TLR1A	FJ477857	4	1	2457	818	92.7
TLR1B	FJ477858	4	1	1959	652	92.6
TLR2A	FJ477860	4	1	2382	793	91.7
TLR2B	FJ477861	4	1	2346	781	93.1
TLR3	ENSMGAG00000011425	4:44877255-44886702	5	2969*	897	81.1
TLR4	ENSMGAG0000005422	19:6407342-6411916	4	2529*	842	92.4
TLR5	ENSMGAG00000015929	2:18293530-18296112	1	2583	860	93.4
TLR7	ENSMGAG00000014706	1:132506616-132510395	3	3331*	1047	91.8
TLR15	ENSMGAG00000015891	2:3159788-3162394	1	2607	868	92.4
TLR21	ENSMGAG00000015581	13:2835516-2838347	1	2919	973	89.8

The accession numbers of NCBI or gene ID of ensemble turkey genome draft are listed. [a] % id- Percentage identity of turkey TLR genes with corresponding chicken ortholog at amino acid level.*Splice variants are found.

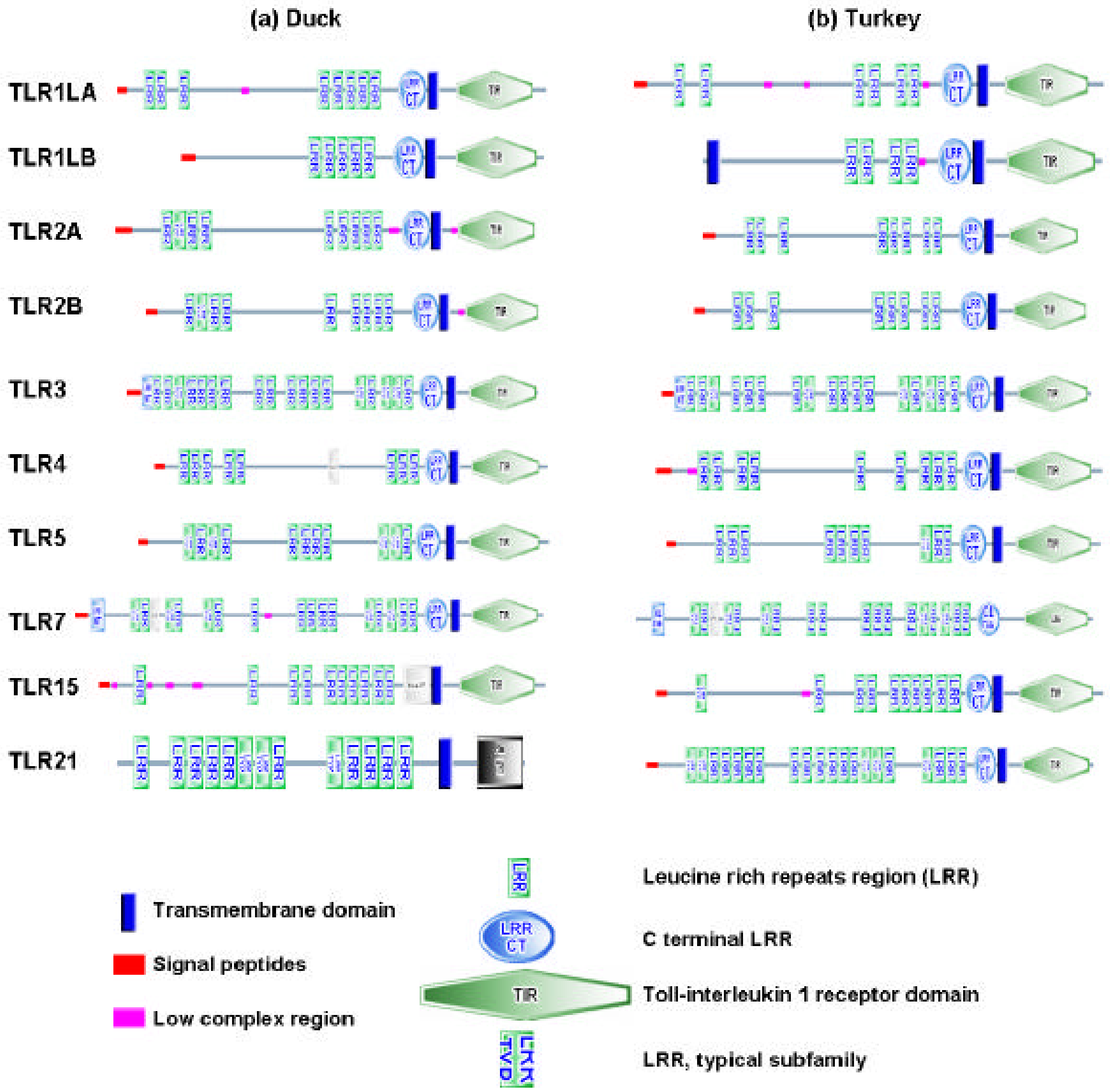


Fig. 4.7 : **Structures of the duck and turkey TLRs**
Available and annotated duck and turkey TLR amino acid sequences were used for analysis
Domains in the protein were predicted by the SMART program

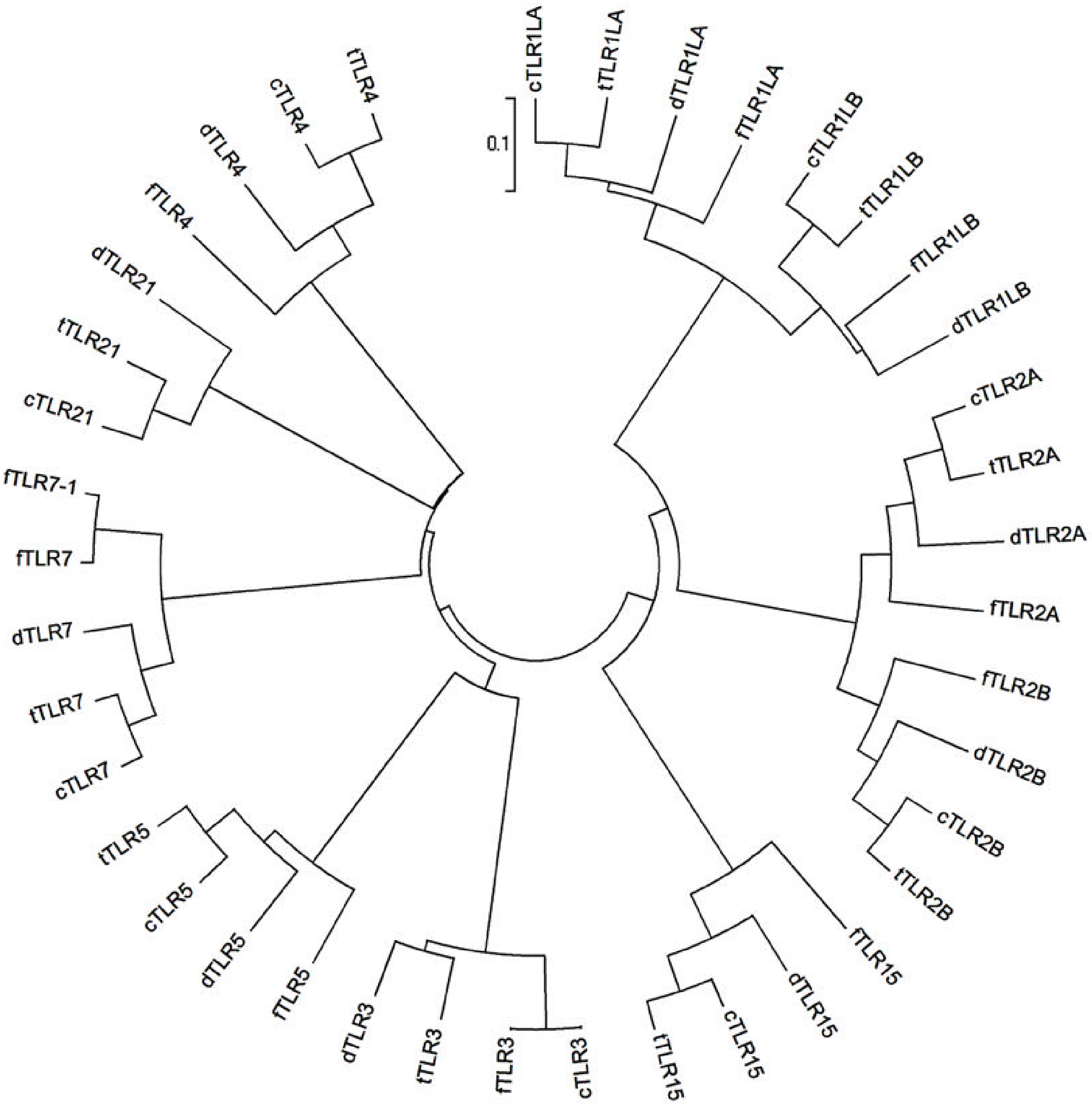
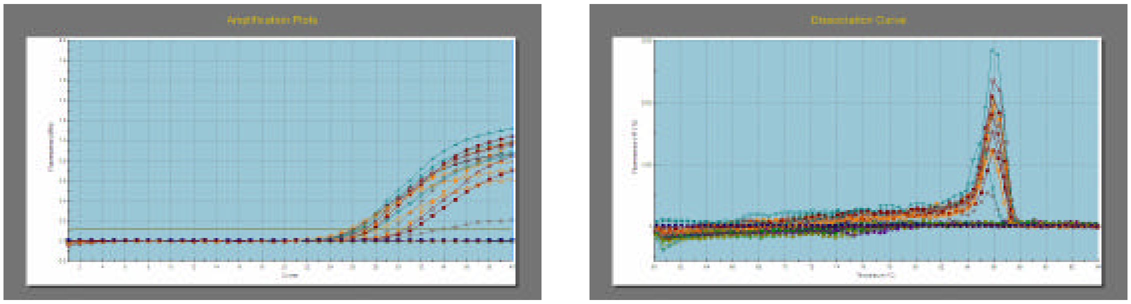


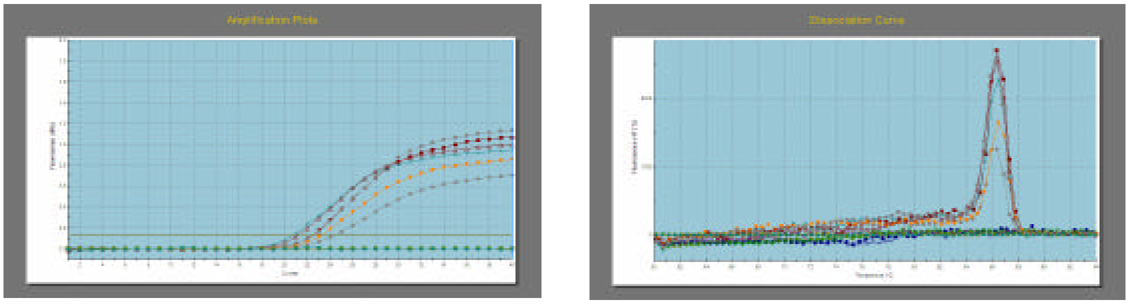
Fig. 4.8 : **Unrooted phylogenetic tree of avian TLRs**

Neighbor joining tree of avian TLRs constructed using full length amino acid sequences using MEGA version 4.0 (Poisson correction model, 1000 bootstrap replicates) cTLR-chicken TLR; dTLR-duck TLR; tTLR- turkey TLR; fTLR- zebra finch TLR; qTLR-Japanese quail TLR.

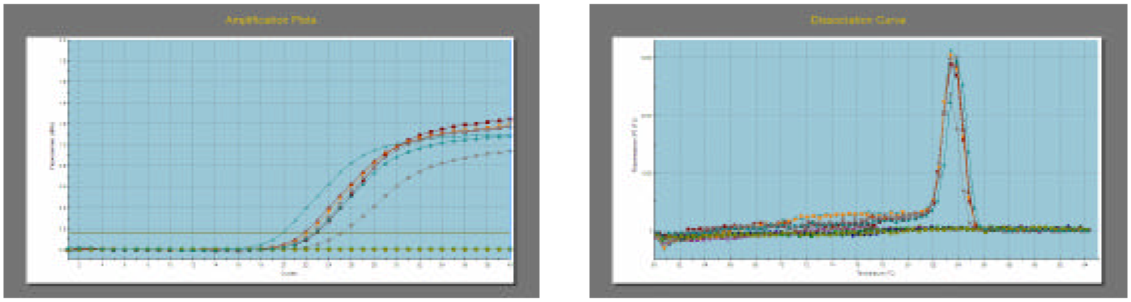
Fig. 4.9 : **Representative amplification plots and dissociation curves of duck *TLR* genes analyzed (a)** *TLR1LA*; **(b)** *TLR1LB*; **(c)** *TLR2A* ; **(d)** *TLR2B*; **(e)** *TLR3*; **(f)** *TLR4*; **(g)** *TLR5*; **(h)** *TLR7*; **(i)** *TLR15*; **(j)** *TLR 21*



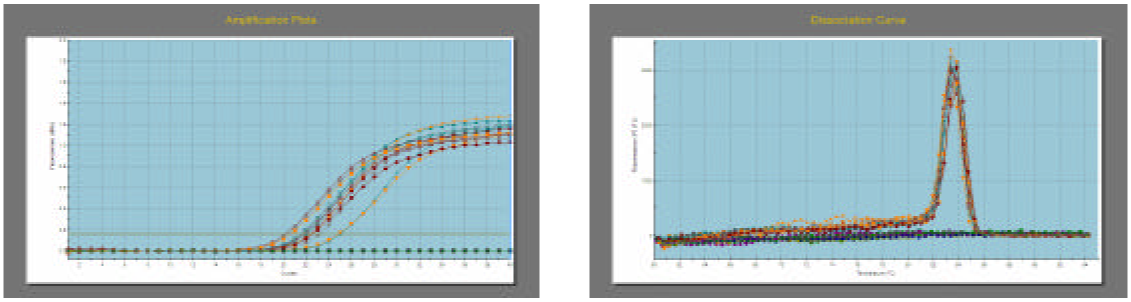
(a)



(b)



(c)



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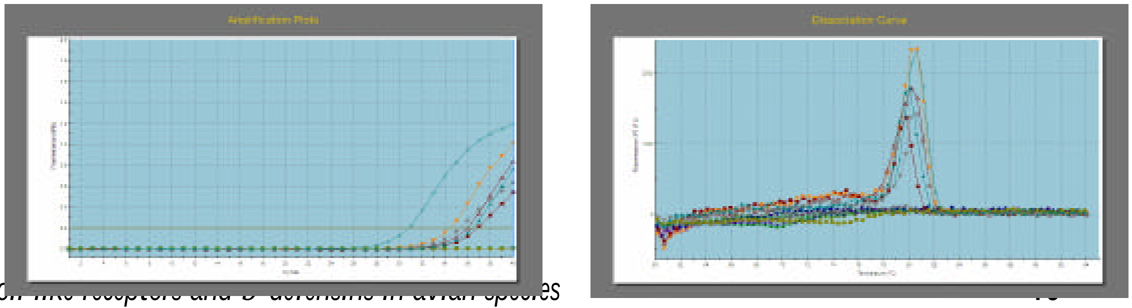

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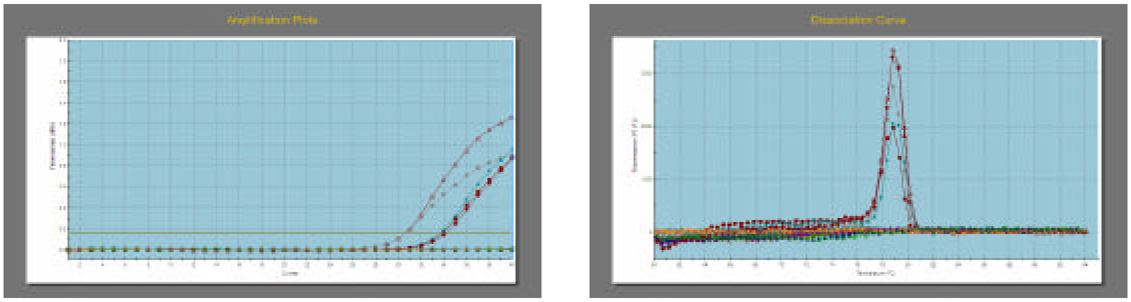
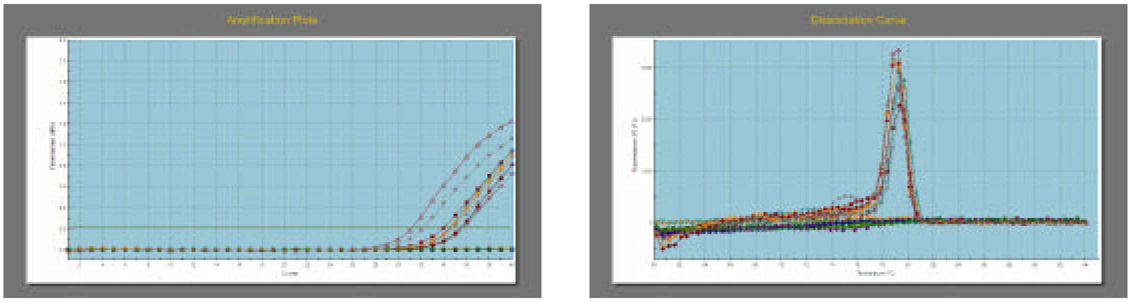
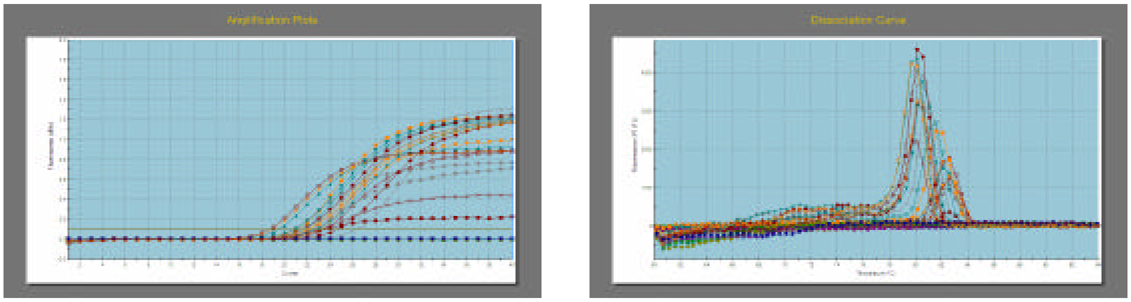

Amplification Plots
Dissociation Curve

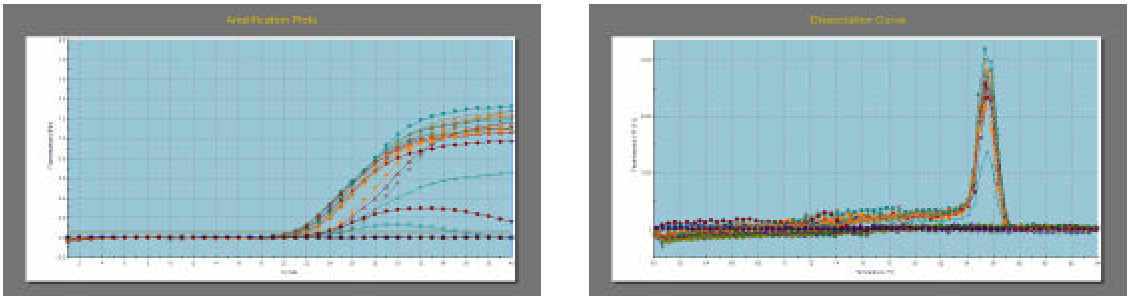
(f)


Amplification Plots
Dissociation Curve

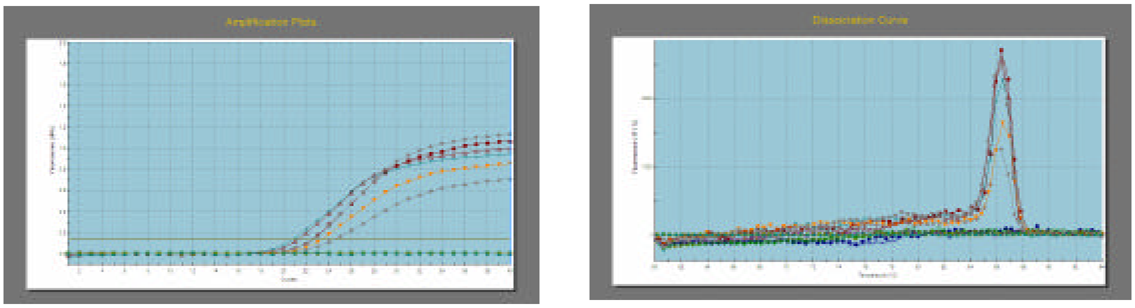
(g)


Amplification Plots
Dissociation Curve

(h)


Amplification Plots
Dissociation Curve

(i)

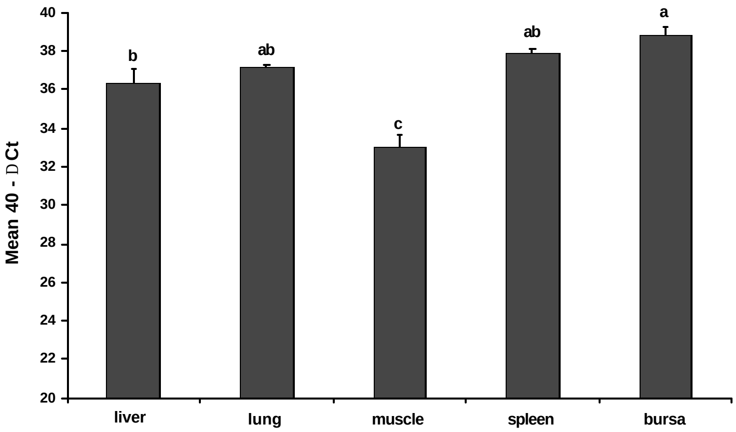

Amplification Plots
Dissociation Curve

(j)

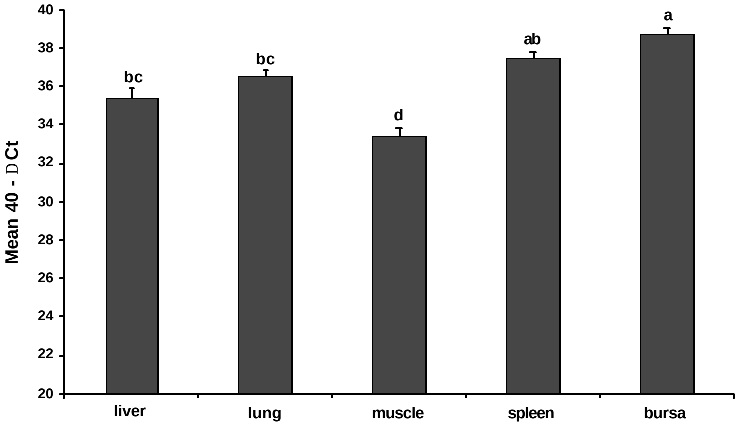
Fig. 4.10 : Differential expression of duck *TLR*s mRNA in different tissues

(a) *TLR1LA*; **(b)** *TLR1LB*; **(c)** *TLR2A* ; **(d)** *TLR2B*; **(e)** *TLR3*; **(f)** *TLR4*; **(g)** *TLR5*; **(h)** *TLR7*; **(i)** *TLR15*; **(j)** *TLR 21*

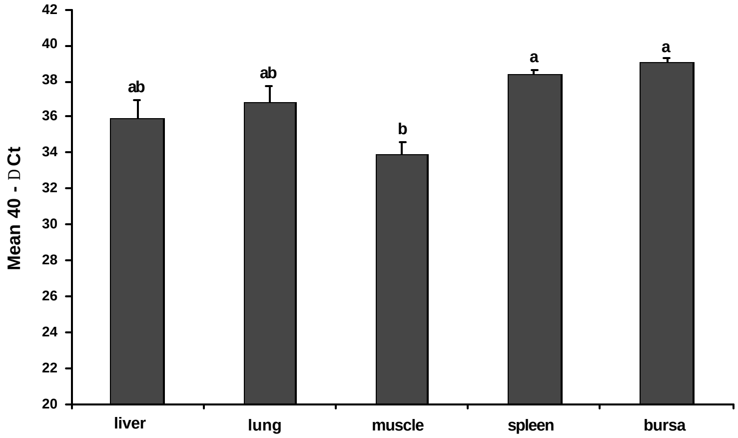
Values are mean 40-ΔCt±SEM (n = 6), Bars with different superscripts differ significantly, P≤ 0.01



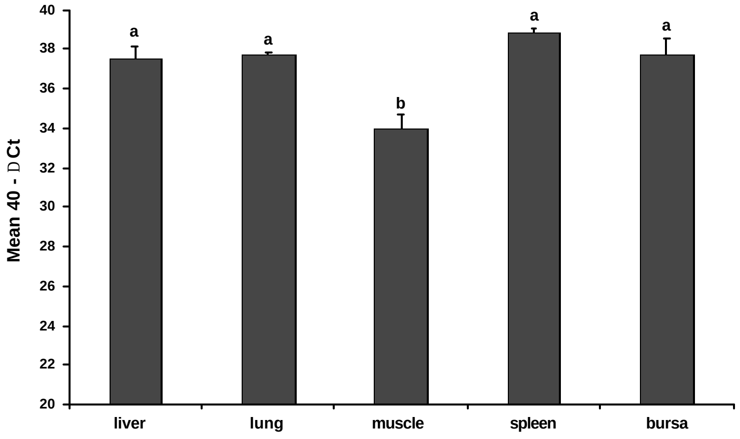
(a) TLR1 LA



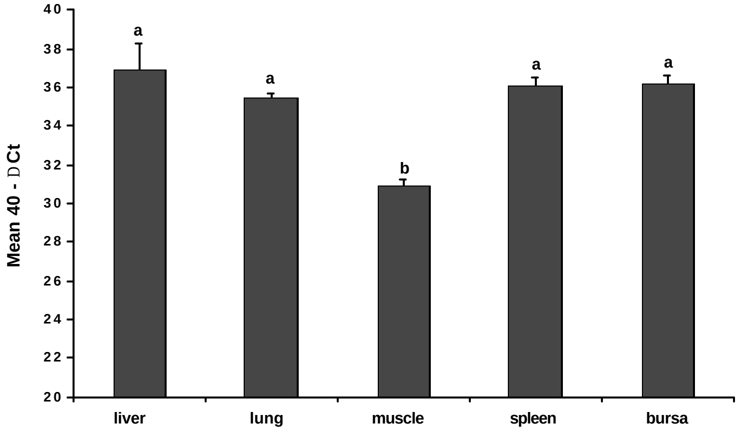
(b) TLR1 LB



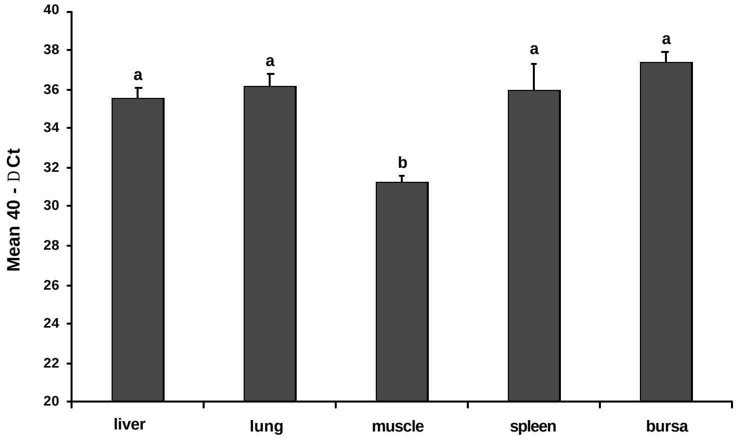
(c) TLR2A



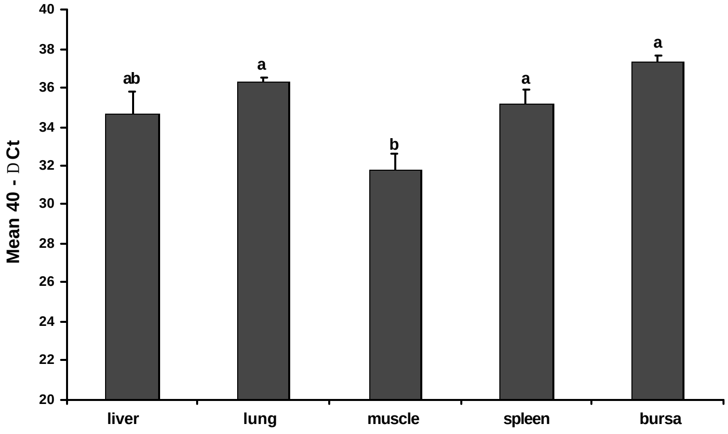
(d) TLR2B



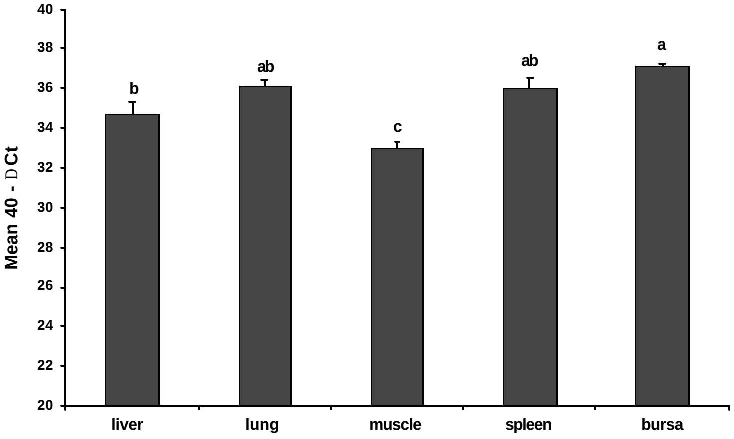
(e) TLR3



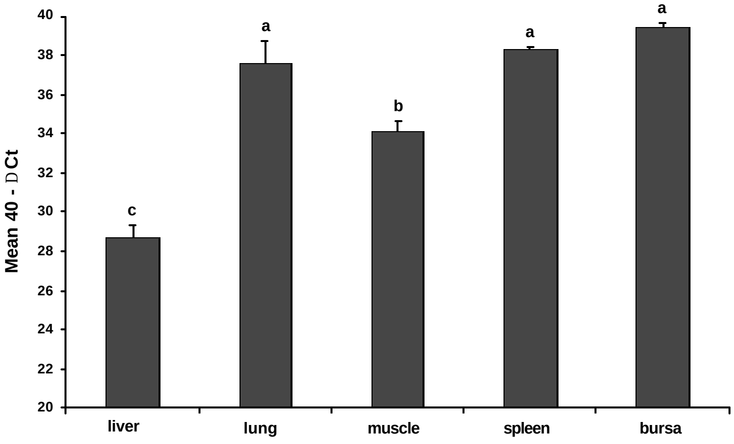
(f) TLR4



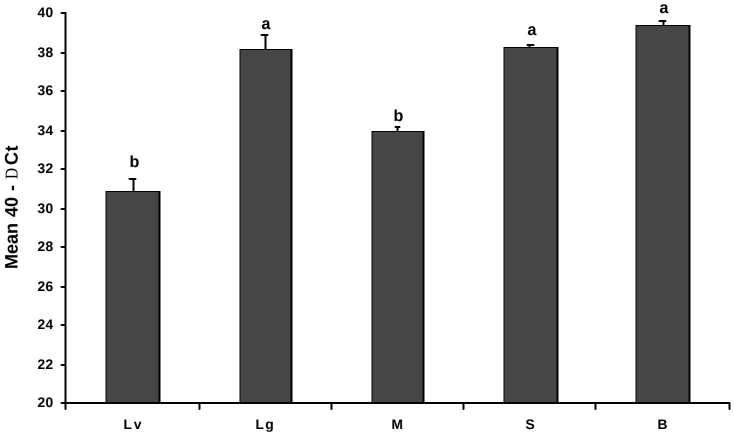
(g) TLR5



(h) TLR7



(i) TLR15



(j) TLR21

4.6.2. Molecular characterization of avian TLR genes

Duck and turkey *TLR* genes showed 77 to 92% similarity at amino acid level to their chicken counter parts. Using the SMART program, typical structures of turkey TLR proteins were predicted (Fig.4.7). Almost all TLR proteins consisted of multiple LRRs in the N-terminal region and a single TIR domain in the C-terminus separated by a transmembrane region except for turkey *TLR7* in which transmembrane domains (TM) could not be detected by the SMART program. Although the SMART program could not predict the transmembrane region in turkey *TLR7*, hydrophobic regions were present between the LRR region and TIR domain. With the help of TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) program TM domain in the hydrophobic region was predicted. The number of LRRs and its phasing showed general conformity with chicken TLR proteins amidst minor variations.

4.6.3. Phylogenetic analysis of avian TLRs

To examine the relationship between avian TLRs (chicken, zebra finch, duck and turkey) a phylogenetic tree was constructed using MEGA program by using all available and annotated avian TLR amino acid sequences (Fig. 4.8). Each of duck and turkey TLR sequences was clustered with its respective chicken and finch TLRs and their bootstrap probabilities were sufficiently high to indicate that these annotations were reliable.

4.6.4. Evolutionary analysis of avian TLRs

Evolutionary analysis of TLR sequences from avian species revealed synonymous substitution (d*S*) was significantly ($P<0.01$) higher than non synonymous substitution (d*N*) for all TLR sequences studied except for duplicated TLR2 genes suggesting that these genes were under purifying selection. The duplicated TLR2 genes of avian are under positive selection.

4.6.5. Expression analysis of duck *TLR* genes

TLR transcripts were detected for all ten genes in all duckling tissue samples analyzed in the present study. Representative amplification plots and dissociation curves of duck *TLR* genes analyzed are given in Fig. 4.9. All ten *TLR*s mRNA expressions were significantly higher in bursa than other tissues studied, whereas in muscle all *TLR*s mRNA expressions were significantly ($P<0.01$) lower except for *TLR15*. *TLR7* gene expression was significantly ($P<0.01$) higher in spleen, bursa and also in lung. In spleen, *TLR5* was least expressed, whereas in bursa *TLR3* was least expressed among all other TLRs investigated (Fig. 4.10).

4.6.6. Expression analysis of turkey *TLR* genes

TLR mRNA expressions were detected for all ten genes in all tissue samples analyzed in the present study (Fig. 4.11). Overall TLR expression was highest for *TLR4* and lowest for *TLR21*. Expression of *TLR1A*, *2A*, *2B* and *21* genes were significantly (P<0.01) higher in liver than other tissues investigated. *TLR3* expression was significantly (P<0.01) higher in marrow (BM) and spleen in comparison to other tissue samples. However, there is no significant difference in the gene expression of *TLR1B, 4, 5, 7* and *15* among the tissues studied (Fig. 4.11).

4.7. Molecular characterization coding region of avian TLR15

4.7.1. Avian TLR15 coding sequence analysis

All five overlapping fragments of TLR15 gene were amplified from spleen cDNA of Aseel, Kadaknath, Japanese quail and turkey (Fig.4.12). The nucleotide sequences of *TLR15* fragments were aligned to generate full-length ORF sequences, which were submitted to NCBI Genbank. Accession numbers were obtained for Aseel (HM773174), Kadaknath (HM773175), Japanese quail (HM773176) and turkey (HQ456924.1). All four coding sequences had same ORF length of 2,607 bp similar to that of broiler chicken (NM_001037835) encoding 868 amino acids, and had molecular weight of 98.2, 98.2, 98.1 and 98.2 kDa, respectively. By using Clustal (W) analysis, Aseel, Kadaknath, Japanese quail, and turkey TLR15 sequences showed 99.7%, 99.8%, 95.1%, and 92.4%, similarity with broiler chicken *TLR15* coding sequence at nucleotide level respectively (Fig. 4.13).

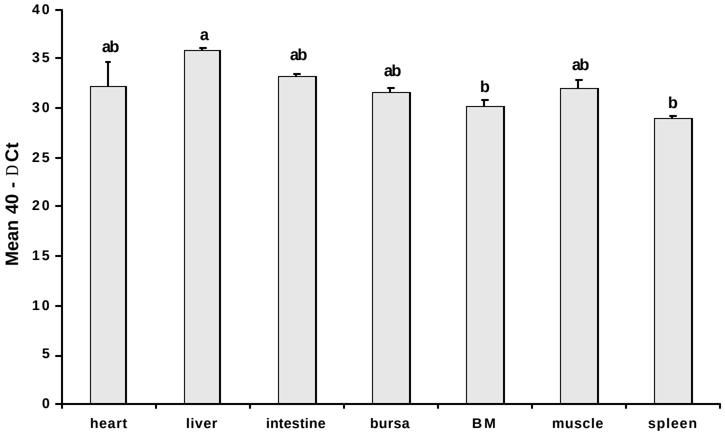
4.7.2. Avian TLR15 protein structure analysis

Structure of TLR15 protein of indigenous chicken breeds, Japanese quail and turkey was predicted based on deduced amino acid sequences from their respective coding sequences using SMART and TMHMM programmes. In all four avian TLR15 sequences first 22 amino acids constituted signal peptide region, followed by ectodomain region that covered over 652 aa residues, transmembrane region (from 654 to position 676) and cytoplasmic TIR domain consisteds of 144 residues (from706 to position 849). SMART prediction revealed that ectodomain of indigenous chickens and turkey TLR15 possesses 10 leucine-rich repeats (LRRs) similar to broiler chicken, whereas Japanese quail has 11 LRRs implying minor differences

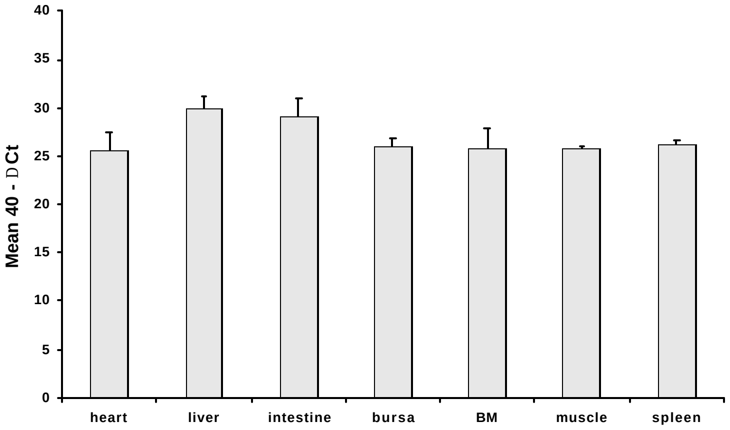
Fig. 4.11 : Differential expression of turkey *TLR*s mRNA in different tissues

(a) *TLR1LA*; **(b)** *TLR1LB*; **(c)** *TLR2A* ; **(d)** *TLR2B*; **(e)** *TLR3*; **(f)** *TLR4*; **(g)** *TLR5*; **(h)** *TLR7*; **(i)** *TLR15*; **(j)** *TLR 21*

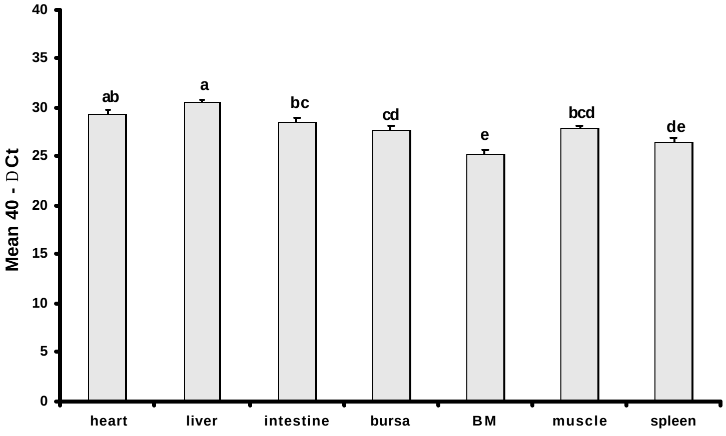
Values are mean 40-ΔCt±SEM (n = 6), Bars with different superscripts differ significantly, $P \leq 0.01$



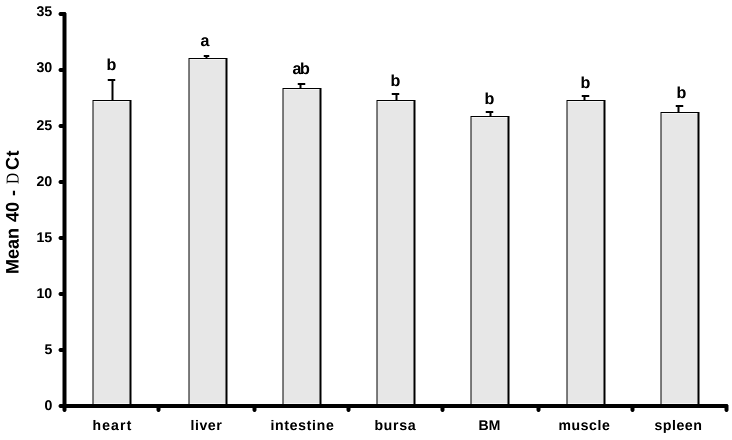
(a) TLR1 LA



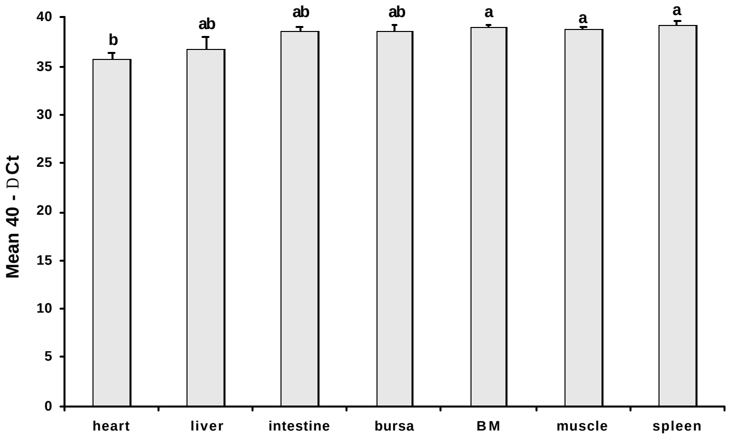
(b) TLR1 LB



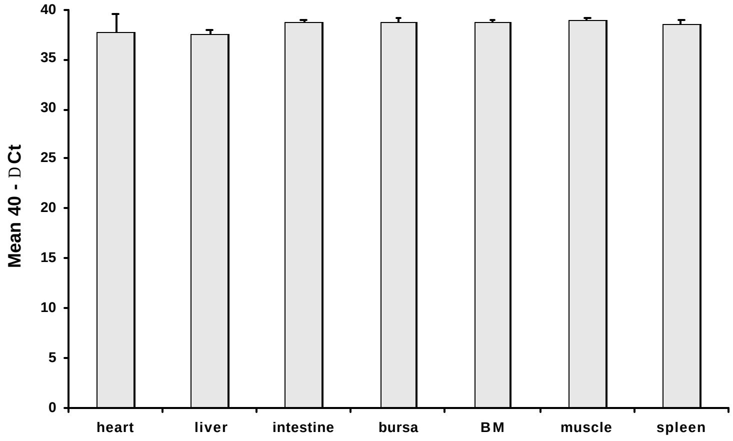
(c) TLR2A



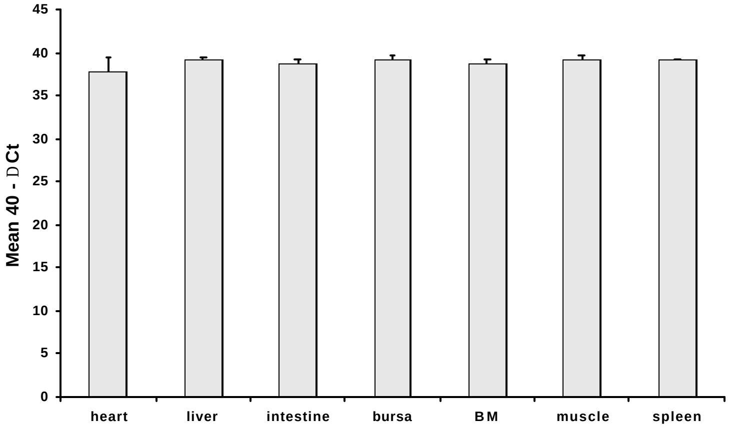
(d) TLR2B



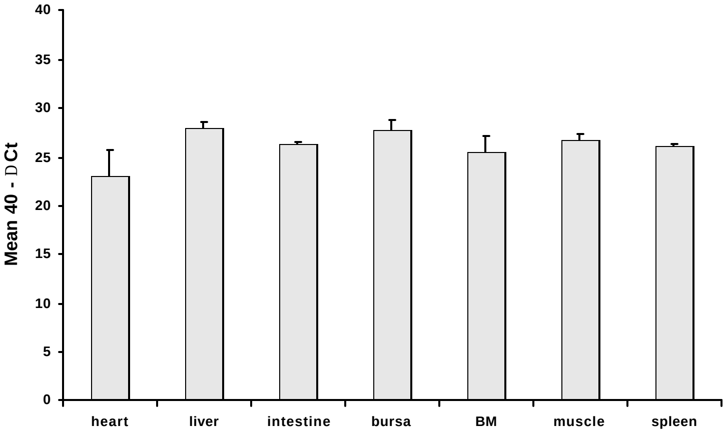
(e) TLR3



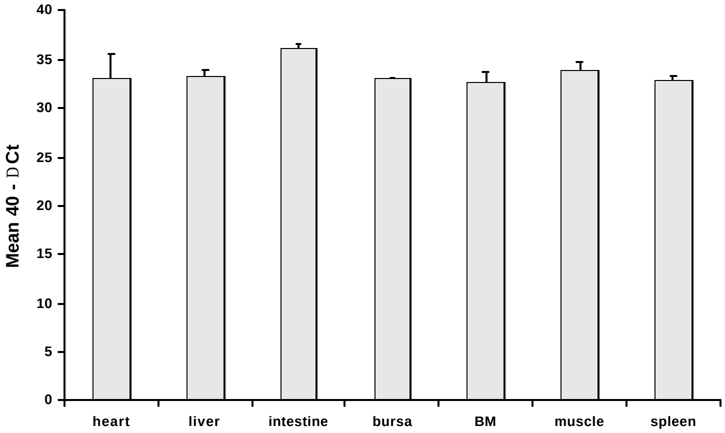
(f) TLR4



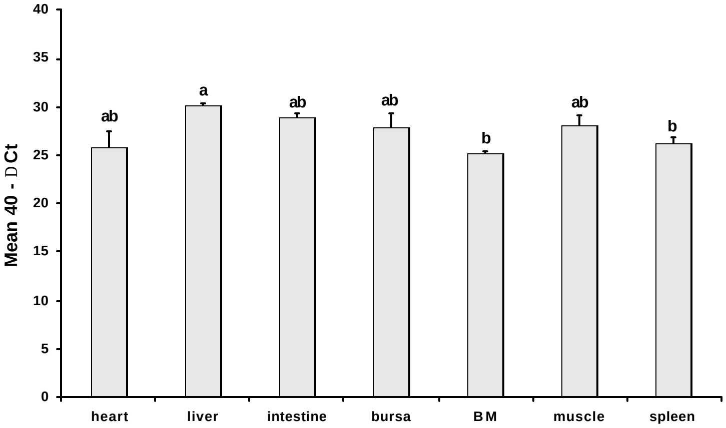
(g) TLR5



(h) TLR7



(i) TLR15



(j) TLR21

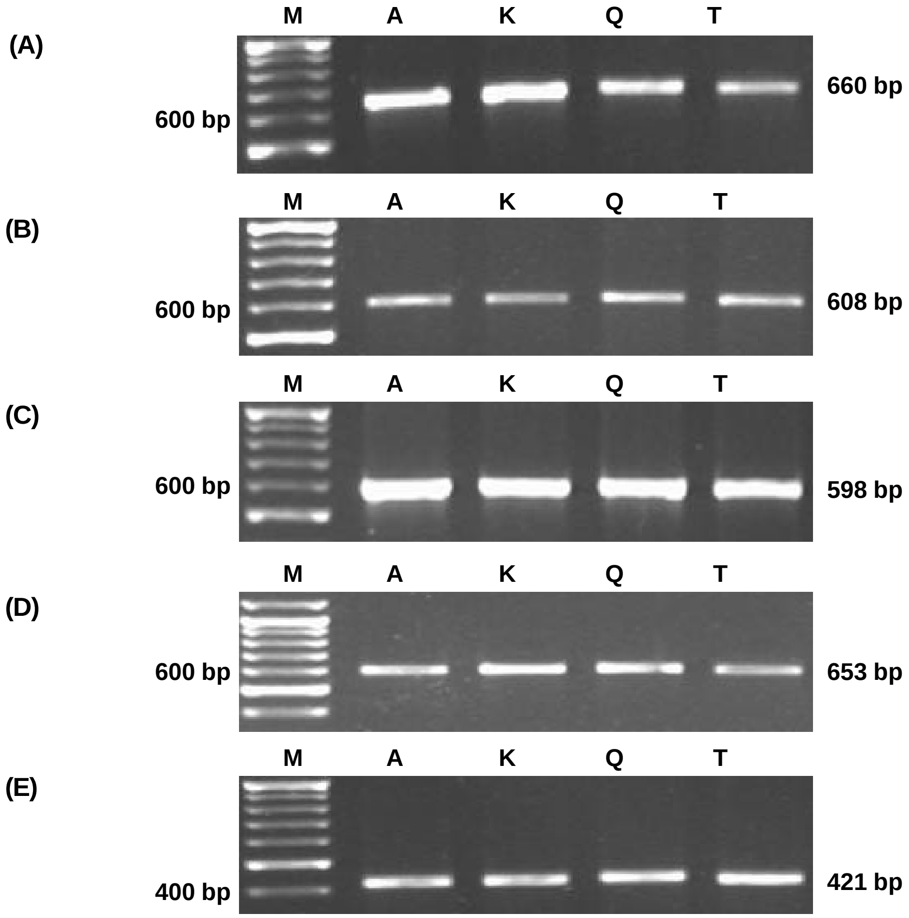


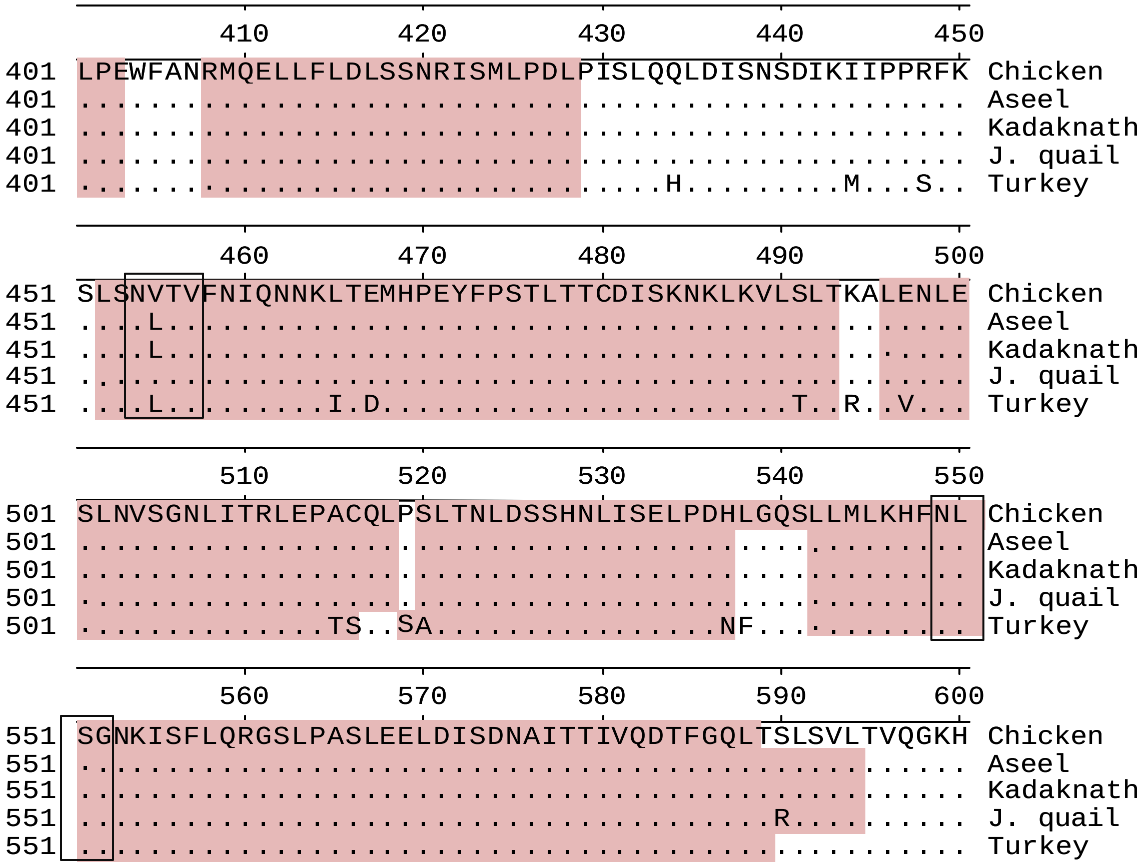
Fig. 4.12 : PCR amplification of fragments of full length coding region of avian TLR15
Lane M : 100 bp DNA ladder**; Lane A :** Aseel**; Lane K :** Kadaknath**; Lane Q :** Japanese quail**; Lane T :** Turkey**, (A)** TLR15-fragment 1**; (B)** TLR15-fragment 2**; (C)** TLR15-fragment 3**; (D)** TLR15-fragment 4**, (E)** TLR15-fragment 5

Fig. 4.13 : Alignment of TLR15 amino acid sequences deduced from full-length ORF sequences of Aseel, Kadaknath, Japanese quail, turkey and broiler chicken. LRRs predicted by SMART are shown as shadowed areas and boxes indicate glycosylation sites predicted by N glycosylation programme. Bold letters in red are the predicted signal sequences and arrows indicate domains predicted with DNA star, PROSITE, TMHMM, and SignalP programmes

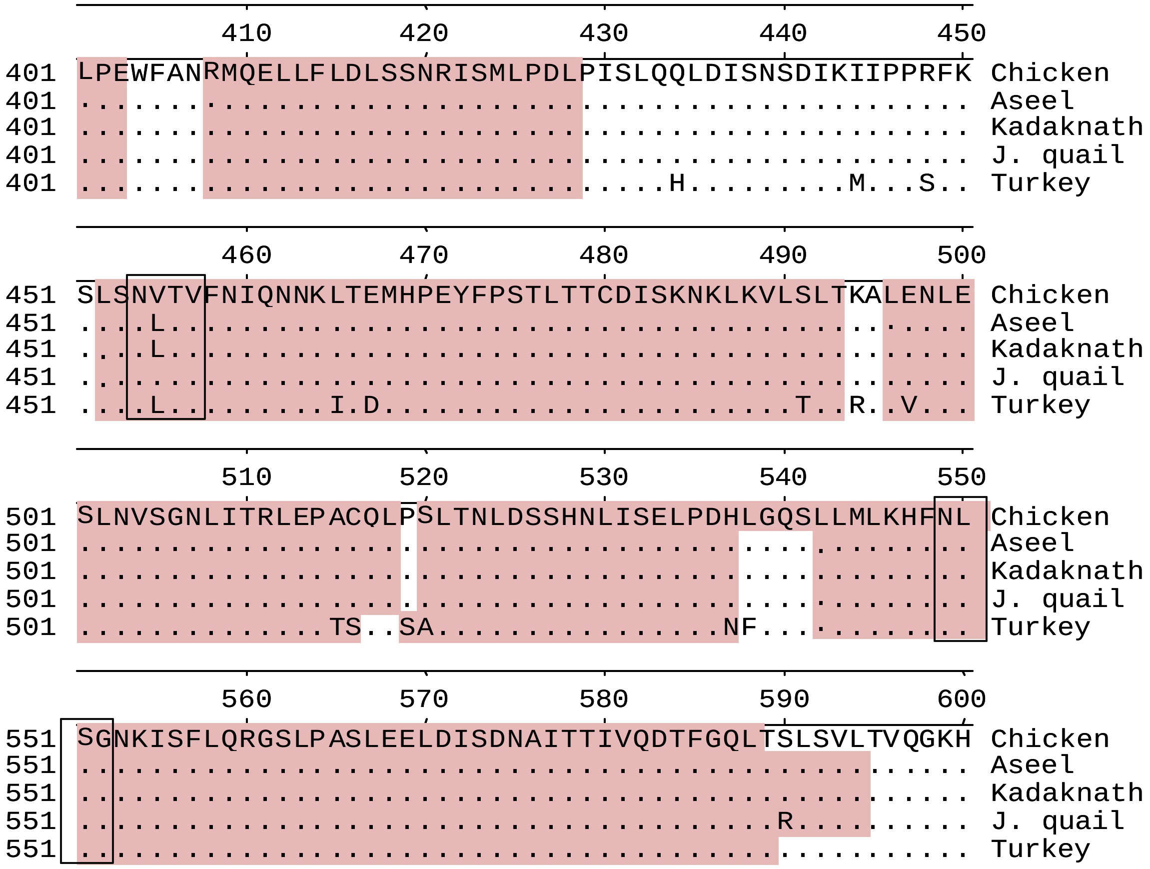
Ectodomain

```
                   10        20        30        40        50
1    MRILIGSLYFYFISFLFSKVNGFLTQRTSPVSSFPFYNYSYLNLSSVSQA Chicken
1    .................................................. Aseel
1    .................................................. Kadaknath
1    ........................I.......................E. J. quail
1    ..V.T....C.......RA.....V.T...M.............T...E. Turkey

                   60        70        80        90       100
51   QAPKTARALNFSYNAIEKITKRDFEGFHVLEVLDLSHNHIKDIEPGAFEN Chicken
51   .................................................. Aseel
51   .................................................. Kadaknath
51   R...........H.......Q......D........Y............. J. quail
51   ............H..............D........Y............. Turkey

                  110       120       130       140       150
101  LLSLVSVDLSFNDKNLLVSGLAPHLKLIPTSGASGPSQIYMYFQKSAEAA Chicken
101  .................................................. Aseel
101  .................................................. Kadaknath
101  ......................AR...KLS....E......L...P.... J. quail
101  .............E......R.S..........L....T..........T Turkey

                  160       170       180       190       200
151  LEPSAPAELLPHLEDPPNPGNVNPRFRQRRTEENKTSPPAATLRPDLCGA Chicken
151  .................................................. Aseel
151  .................................................. Kadaknath
151  .......K........SD....R.GL.R..S...T....E........A. J. quail
151  .V..T...........S.....S.......A...T...........V... Turkey

                  210       220       230       240       250
201  PINGLLDLSRTKLSNEELTAKLDADLCQAQLGTVLEFNISHSDLEMDLLS Chicken
201  .................................................. Aseel
201  .................................................. Kadaknath
201  .........G......................A................. J. quail
201  ..............D...A..........K..A................. Turkey

                  260       270       280       290       300
251  LFILFLPMKDIQSVDASYNRITINNIDVEAICHFPFSNFSFLNISNNPIN Chicken
251  .................................................. Aseel
251  .................................................. Kadaknath
251  .................N.K.............................. J. quail
251  ...........R...................................... Turkey

                  310       320       330       340       350
301  SLETVCLPASITVIDLSFTNISTIPANFAKKLSKLERMYVQGNQLIYTVR Chicken
301  ...............................F.................. Aseel
301  ...............................F.................. Kadaknath
301  ....M...PT........................................ J. quail
301  R.......PT.R........................C............. Turkey

                  360       370       380       390       400
351  PENPSATPRPPPGTVQISAISLVRNQAGTPIESLPESVKHLKVSNCSIVE Chicken
351  .................................................. Aseel
351  .................................................. Kadaknath
351  ..SS......L....R.....F............................ J. quail
351  ..SS..AT.......H.....F..............R.....A....... Turkey
```

Contd...



```
                     410       420       430       440       450
401 LPEWFANRMQELLFLDLSSNRISMLPDLPISLQQLDISNSDIKIIPPRFK Chicken
401 .................................................. Aseel
401 .................................................. Kadaknath
401 .................................................. J. quail
401 .................................H.........M...S.. Turkey

                     460       470       480       490       500
451 SLSNVTVFNIQNNKLTEMHPEYFPSTLTTCDISKNKLKVLSLTKALENLE Chicken
451 ....L............................................. Aseel
451 ....L............................................. Kadaknath
451 .................................................. J. quail
451 ....L.........I.D.......................T..R..V... Turkey

                     510       520       530       540       550
501 SLNVSGNLITRLEPACQLPSLTNLDSSHNLISELPDHLGQSLLMLKHFNL Chicken
501 .................................................. Aseel
501 .................................................. Kadaknath
501 .................................................. J. quail
501 ..............TS..SA................NF............ Turkey

                     560       570       580       590       600
551 SGNKISFLQRGSLPASLEELDISDNAITTIVQDTFGQLTSLSVLTVQGKH Chicken
551 .................................................. Aseel
551 .................................................. Kadaknath
551 .......................................R.......... J. quail
551 .................................................. Turkey
```



```
                     410       420       430       440       450
401 LPEWFANRMQELLFLDLSSNRISMLPDLPISLQQLDISNSDIKIIPPRFK Chicken
401 .................................................. Aseel
401 .................................................. Kadaknath
401 .................................................. J. quail
401 .................................H.........M...S.. Turkey

                     460       470       480       490       500
451 SLSNVTVFNIQNNKLTEMHPEYFPSTLTTCDISKNKLKVLSLTKALENLE Chicken
451 ....L............................................. Aseel
451 ....L............................................. Kadaknath
451 .................................................. J. quail
451 ....L.........I.D.......................T..R..V... Turkey

                     510       520       530       540       550
501 SLNVSGNLITRLEPACQLPSLTNLDSSHNLISELPDHLGQSLLMLKHFNL Chicken
501 .................................................. Aseel
501 .................................................. Kadaknath
501 .................................................. J. quail
501 ..............TS..SA................NF............ Turkey

                     560       570       580       590       600
551 SGNKISFLQRGSLPASLEELDISDNAITTIVQDTFGQLTSLSVLTVQGKH Chicken
551 .................................................. Aseel
551 .................................................. Kadaknath
551 .......................................R.......... J. quail
551 .................................................. Turkey
```

Contd...

```
             610       620       630       640       650
601 FFCNCDLYWFVNIYIRNPHLQINGKDDLRCSFPPDRRGSLVKSSNLTLLH Chicken
601 .................................................. Aseel
601 .................................................. Kadaknath
601 ............V..H..........................G....... J. quail
601 ............V..H.................................. Turkey
      →     TM domain   ←
             660       670       680       690       700
651 CSLGIQMAITACMAILVVLVLTGLCWRFDGLWYVRMGWYWCMAKRRQYKK Chicken
651 .................................................. Aseel
651 .................................................. Kadaknath
651 ............V.V..............................K.... J. quail
651 .............................................K.... Turkey
       ———————→      TIR domain
             710       720       730       740       750
701 RPENKPFDAFISYSEHDADWTKEHLLKKLETDGFKICYHERDFKPGHPVL Chicken
701 .................................................. Aseel
701 .................................................. Kadaknath
701 ..................E....N.......................... J. quail
701 .......................N.......................... Turkey

             760       770       780       790       800
751 GNIFYCIENSHKVLFVLSPSFVNSCWCQYELYFAEHRVLDENQDSLIMVV Chicken
751 ............D..................................... Aseel
751 ............D..................................... Kadaknath
751 ..............................................L.I. J. quail
751 ................................................I. Turkey

             810       820       830       840       850
801 LEDLPPDSVPQKFSKLRKLLKRKTYLKWSPEEHKQKIFWHQLAAVLKTTN Chicken
801 .................................................. Aseel
801 .................................................. Kadaknath
801 ......N.............................M............. J. quail
801 ......N........................................... Turkey

             860
851 EPLVRAENGPNEDVIEME                                 Chicken
851 ..................                                 Aseel
851 ..................                                 Kadaknath
851 ........E.........                                 J. quail
851 .............I....                                 Turkey
       ———————→
       TIR domain
```

across the species (Fig.4.14). There are no N-terminal cysteine clusters in TLR15 in all four sequences studied.

4.7.3. Phylogenetic analysis of avian TLR15

Based on the amino acid sequences of TLR15, phylogenetic tree was drawn by MEGA 3.1 considering 1,000 bootstrap values including all chicken TLR sequences available zebra finch (*Taeniopygia guttata*) TLR sequences and TLR15 sequences of Aseel, Kadak Japanese quail and turkey (Fig. 4.15). Avian TLR15 sequences grouped with chicken and finch TLR1 family with high bootstrap support.

4.8. Functional characterization of duck TLRs

4.8.1. Cytokine mRNA expression in duck PBMC culture stimulated with TLR agonists in *in vitro* assay

IL-1ß gene expression level in LPS stimulated duck PBMC culture were significantly higher at both 12 h (1.9 fold change; $P<0.05$) and 24 h (2.16 fold change; $P<0.01$) time intervals. However, there were no significant changes in IFN-? gene expression levels in poly I:C stimulated duck PBMC culture at both 12 h (0.60 fold change) and 24 h (0.53 fold change) intervals (Table 4.8).

Table 4.8. Cytokines IL-1ß and IFN-? gene expression levels TLR agonists stimulated duck PBMC culture

TLR agonist	**LPS**[a]		**Poly I:C**[b]	
Time interval	**12**	**24**	**12**	**24**
Control (mean 40-ΔCt)	31.34±0.17	32.33±0.33	38.95±0.28	39.11±0.28
Stimulated culture (mean 40-ΔCt)	35.04±0.38	34.5±0.09	39.55±0.20	39.64±0.05
Fold change $(2^{-\Delta\Delta Ct})$	1.9*	2.16**	0.60	0.53

[a]: IL-1ß,[b]: IFN-? gene expression levels * Significant at $P<0.05$, ** Significant at $P<0.01$. n=3 for each experiment.

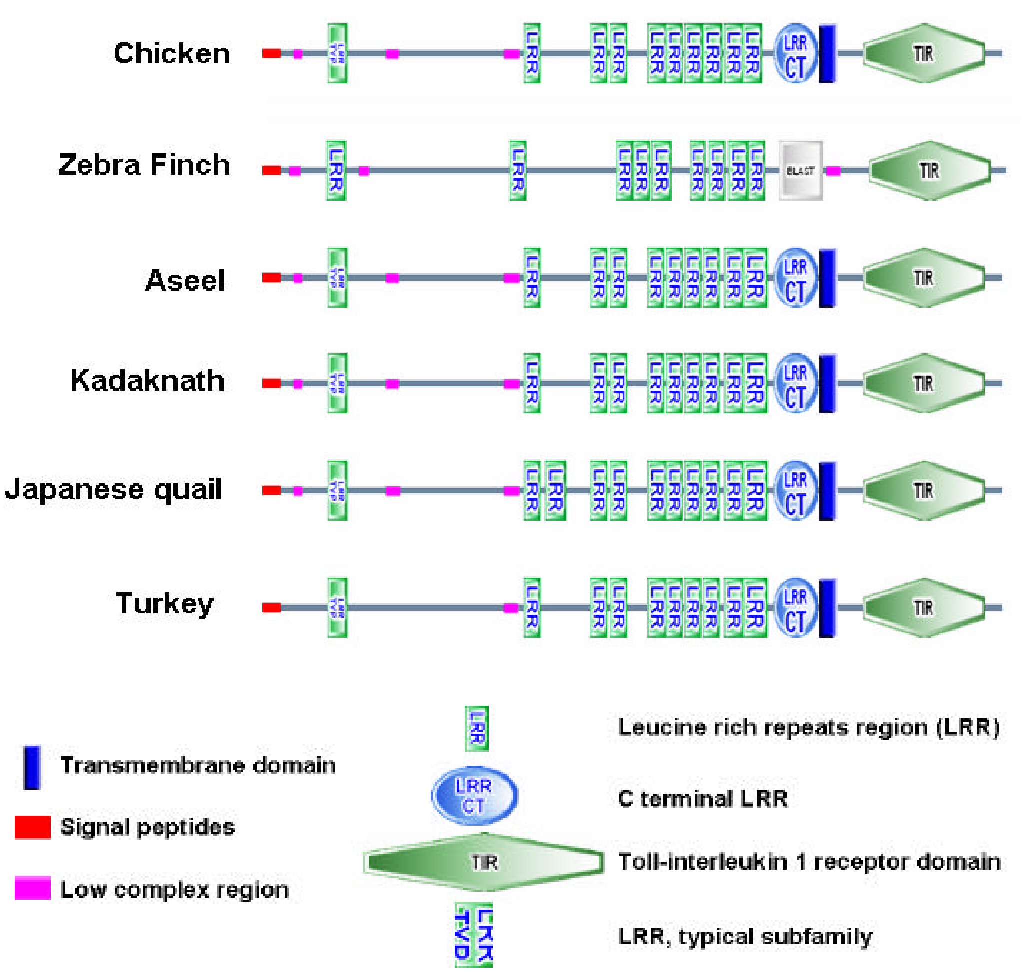


Fig. 4.14 : **Structures of the avian TLR15 proteins**

Domains in the protein were predicted by the SMART program

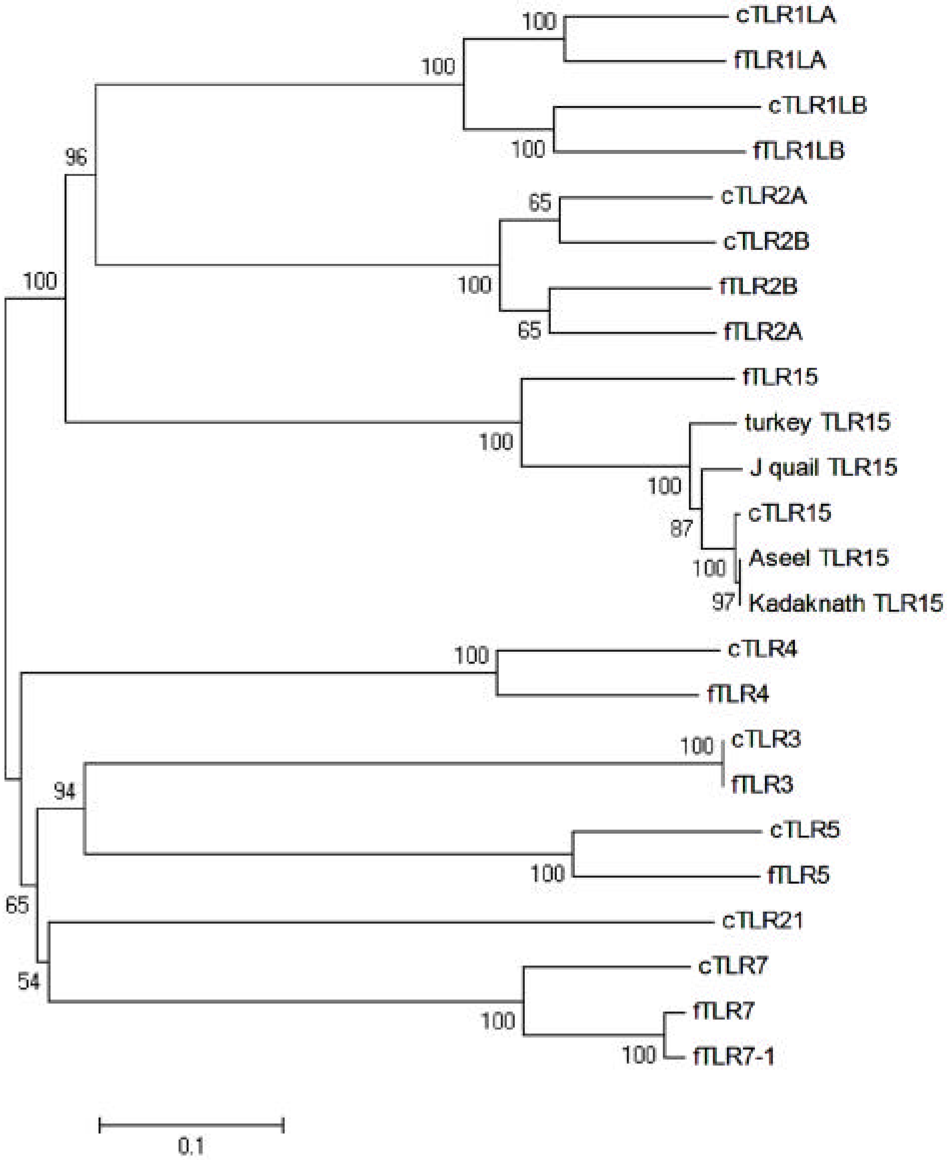


Fig. 4.15 : Phylogenetic relationship of avian TLR15 with other avian TLR sequences

Phylogenetic tree constructed using Mega version 3.1 following alignment of the amino acid sequences using Clustal W and neighbor-joining method (poisson correction model with pairwise deletion option). Numbers outside the branches indicate the bootstrap values obtained using 1,000 replicates and only values above 50% are shown. The scale bar at the bottom measures the distance. c-chicken, f- Zebra finch

4.9. Cloning, prokaryotic expression and purification of recombinant chicken b-defensin-AvBD2

4.9.1. Amplification and cloning of chicken *AvBD2* gene

A 143 bp (119 bp of target gene+primer sequence with restriction enzymes (RE) sites PCR product of chicken *AvBD2* gene was amplified from chicken genomic DNA (Fig. 4.16). The PCR product was cloned into pUC29 cloning vector by RE digestion and ligation. Positive clones checked by *EcoRI* and *Hind III* RE digestion released ~250bp insert from the 2.5Kb cloning vector (Fig. 4.17). Sequence analysis of cloned insert showed 100% similarity with published chicken AvBD2 coding sequence (AF033336).

4.9.2. Prokaryotic expression and purification of chicken AvBD2

The insert was sub cloned in pET-28A expression vector and positive clone was selected for induction of recombinant protein. Induction of the recombinant chicken AvBD2 did not affect the viability of *E. coli* cells transformed with pET-28a-Gga_AvBD2 constructs. SDS-PAGE analysis of the bacterial pellet obtained from cultures transformed with recombinant plasmid showed presence of a 7.7 kDa additional protein (Fig. 4.18). No toxic effect of the protein was observed in the un-induced and induced cultures during the 5 h growth period studied. The growth kinetics of the un-induced cultures were similar in both transformed and control cells. In induced cultures, the overall growth was low; however, the bacterial growth trends in the control and transformed cells were not significantly different.

Analysis of the supernatants and pellet fraction of bacterial cells after disruption by sonication indicated that all the recombinant chicken AvBD2 peptide was present in the pellet fraction in an insoluble form. So a purification scheme using denaturing conditions was employed and the protein was purified to more than 90% purity as evidenced by the coommasie staining of the SDS-PAGE gels after electrophoresis (Fig. 4.19). The yield of the purified protein was about 1-2 mg from a litre of induced *E. coli* culture.

4.8.2. Antibacterial activity of recombinant chicken AvBD2

The recombinant chicken AvBD2 showed antibacterial activity against *S*. Pullorum. The minimum bactericidal concentration (MBC) of recombinant chicken AvBD2 evaluated by micro-broth dilution assay was 35 µg/ ml (Fig. 4.20).

* * *

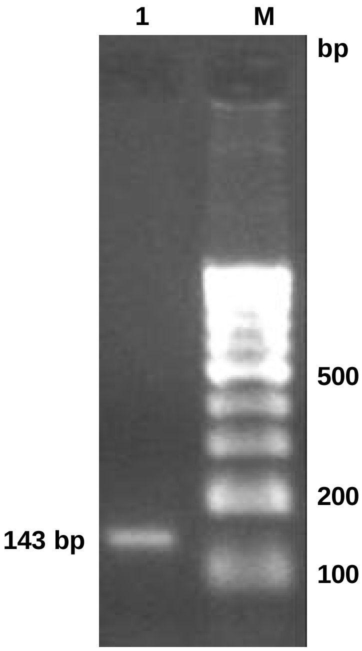


Fig. 4.16 : **PCR amplification of exon 3 of chicken AvBD2 mature peptide gene region**

Lane M	**:**	100 bp DNA ladder
Lane 1	**:**	PCR product

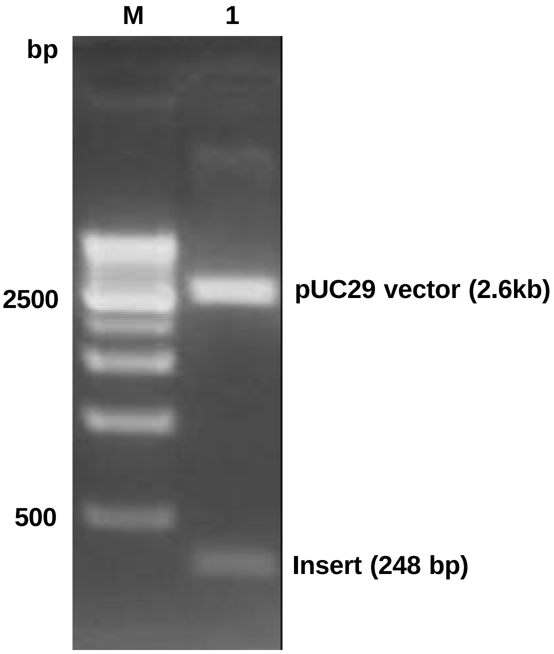


Fig. 4.17 : **Restriction enzyme analysis of recombinant pUC29 cloning vector**

Lane M	**:**	100 bp DNA ladder
Lane 1	**:**	*Eco*RI/ *Hind*III digested recombinant pUC29 vector and insert (248bp)

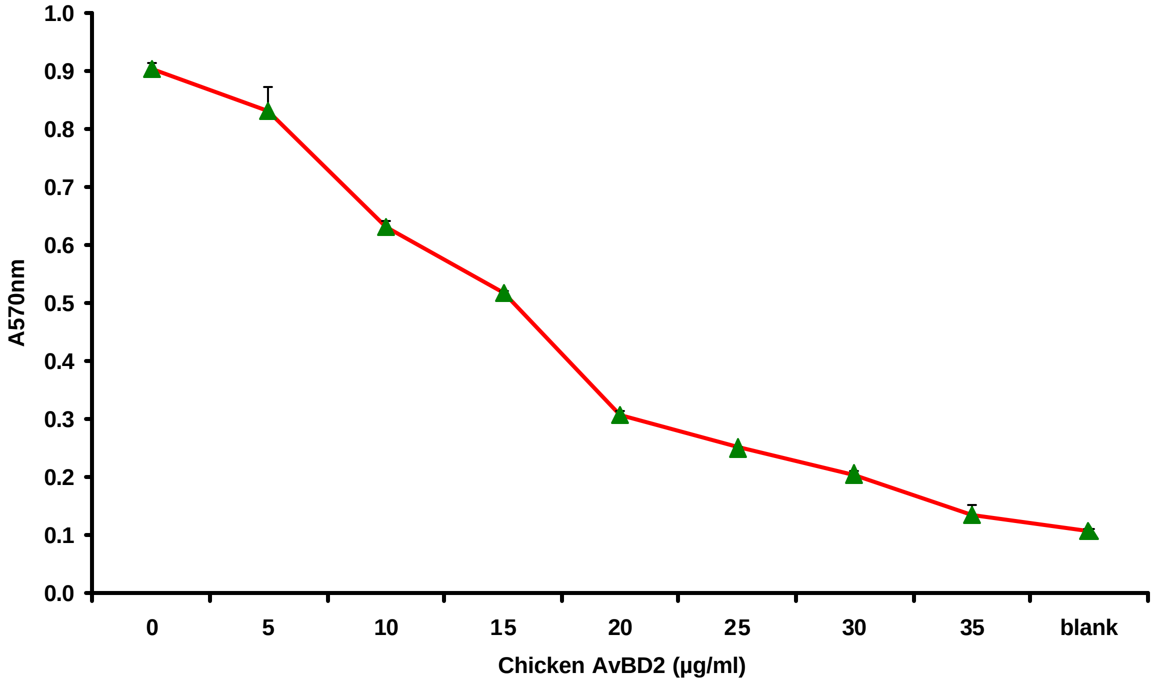


Fig. 4.20 : **Antimicrobial activity of recombinant chicken AvBD2 against *S.* Pullorum by microbroth dilution assay**

5 Discussion

Innate immune system is now considered as potent activator and mediator of the adaptive immune response. Instead of selecting chickens for resistance to a single pathogen, focus on innate biomarkers which indicate potential to mount most effective immune responses against multiple microorganisms would be rather more beneficial. The toll-like receptors (TLR) and ß-defensin super family represents evolutionarily conserved members of innate immune system that can exploited in future in breeding programmes to improve disease resistance and for immunomodulation.

Comparative quantification of constitutive TLRs mRNA expression in Indian native and White Leghorn chickens

Indigenous chicken breeds are considered to be disease resistant, however the underlying immunological basis is unknown. The expression profiles of *TLR*s, their functions and signaling pathways have been elucidated in various mammalian immune cell types (Muzio and Mantovani, 2001; Muzio *et al.,* 2000; Janeway and Medzhitov, 2002 and Takeda and Akira, 2004). Constitutive *TLR* expression profiles are suggestive of an individual's ability to respond to pathogen encounter. Moreover, wider repertoire of TLRs in tissues that interface between host and pathogen could be attributed to greater host resistance (Kogut *et al.,* 2005a; Iqbal *et al.,* 2005a). Expression pattern of *TLR*s in White Leghorn chicken have been earlier documented (Kogut *et al.,* 2005a; Iqbal *et al.,* 2005a), however, there is dearth of information regarding native chickens. Hence, in the present study, TLR gene expression in two Indian native chickens (Aseel and Kadaknath) was investigated and compared with White Leghorn chicken.

Significantly higher levels of *TLR4* mRNA expression in Aseel and White Leghorn were observed in comparison to Kadaknath in different tissues investigated. TLR4 is the principal receptor for LPS, a major component of outer membrane of Gram-negative bacteria (Poltorak *et al.,* 1998). TLR4 is required for LPS mediated inducible nitric oxide synthase (*iNOS*) induction in chicken macrophages (Dil and Qureshi, 2002). Variable expression of TLR4 molecules has been reported previously in different genotype chicken (Dil and Qureshi, 2002). Moreover allelic variations in *TLR4* gene are associated with resistance/susceptibility to *Salmonella enterica* serovar Typhimurium and *Salmonella enteritidis* (SE) infections in chicken (Leveque *et al.,* 2003; Dil and Qureshi, 2002; Malek *et al.,* 2004). However, significantly higher levels of *TLR4* mRNA expression levels in heterophils of Kadaknath were observed in comparison to Aseel, White Leghorn, naked neck and dwarf gene lines (Kannaki *et al.*, 2010a). The discrepancy between these studies may be due to difference in the tissue/ cell types studied (heterophils vs. tissue) and age (adult vs. day-old chicks). Hence the significantly higher expression of *TLR4* in Aseel and White Leghorn birds has implication in nitric oxide production and innate immunity. *TLR7* gene expression was found to be significantly higher in liver of White Leghorn than native chicken tissues (Table 4.1). Differential expression of *TLR7* in variety of tissues has been reported earlier (Kogut *et al.,* 2005a; Iqbal *et al.,* 2005a). *TLR15* and *21* gene expressions were significantly higher in Aseel tissues (spleen and bursa & heart) in comparison to Kadaknath and White Leghorn tissues. Higher and wider expression of *TLR* genes in native chickens may provide edge over their commercial counterparts, which can be exploited in breeding programs to improve the disease resistance in poultry stocks.

Differential *TLR*s mRNA expression patterns during chicken embryological development

In the present study an attempt was made to profile the expression pattern and kinetics of gene expression of *TLR* genes during chicken embryo development. All seven *TLR* (*2A, 3, 4, 5, 7, 15* and *21*) genes were expressed in whole embryonic tissue as early as 3rd embryonic day (ED). Quantitative real-time PCR analysis revealed *TLR* genes are differentially expressed during chicken embryo development and each *TLR* gene showed significant changes in expression suggestive of developmental regulation (Fig. 4.4). The present finding are consistent with the recent findings of expression of innate immune genes in early embryonic tissues (Meade *et al.,* 2009a; Michailidis *et al.,* 2010). In similarity to the earlier finding of *TLR2* gene expression in early embryonic tissues (Meade *et al.,* 2009a), relatively low level of differential expression of *TLR2* was observed in embryonic liver and intestine, whereas declining trend of *TLR2* expression was found in brain and heart tissue during development. Chicken TLR2 has been shown to act as a receptor for lipoprotein and recognizes LPS in the presence of MD-2 similar to human TLR2 (Fukui *et al.,* 2001). *TLR2* is expressed and functional in the developing mouse telencephalon from early embryonic stages and infectious agent-related activation of TLR2 inhibits neuronal progenitor cell (NPC) proliferation (Okun *et al.,* 2010). TLR2-mediated inhibition of NPC proliferation may therefore be a mechanism by which infection, ischemia, and inflammation adversely affect brain development. Some TLRs are strongly expressed in the human embryonic brain and TLR3 and TLR8 have been implicated in neurogenesis and neurite outgrowth in the developing brain, whereas TLR2 and TLR4 have been shown to regulate adult neurogenesis (Mallard *et al.,* 2009).

In contrary to the weak expression of *TLR4* in early embryonic development by Meade *et al.* (2009) (whole embryonic tissue) and Kannaki and Verma (2008) (liver tissue), in the present study strong expression of its transcript in liver during 12th ED was observed. Differential expression of *TLR2* and *TLR4* in developing fetal sheep lung has been shown earlier (Meyerholz *et al.,* 2006; Hillman *et al.,* 2008). TLR2 and TLR4 protein were expressed from the first cell stage through the blastocyst stage of murine embryo (Rose *et al.,* 2011). TLR4, a well known receptor for LPS together with lysozyme, a major protein component of egg known to bind and detoxify LPS (Takada *et al.,* 1994) may provide protection against major Gram negative bacteria in the event of *in ovo* infection. Recently it has been demonstrated that the expression of functional TLR4 is associated with proliferation and maintenance of stem cell phenotype in endothelial progenitor cells (EPCs) (He *et al.,* 2011). This suggests the role of TLR4 in angiogenesis and reveals a novel aspect of the multiple-faced TLR biology, and may open new prospects for using TLR4 agonists clinically. TLR2 (Vora *et al.,* 2004) and TLR4 (Diamond *et al.,* 1996) activated immune pathways have been previously shown to induce antimicrobial peptides. Further recent studies suggested that avian ß-defensins-2 (AvBD2) and AvBD13 may act as endogenous ligand for TLR4 and that AvBD13 enhances the proliferation of monocytes via the NF-?B pathway (Yang *et al.,* 2010). However, an earlier study by Meade *et al.* (2009a) showed both *AvBD2* and *13* were decreased in gene expression during early stages of development in relation to 3rd ED. The complex interlink between *TLR* gene expression and antimicrobial peptide (AMP) expression is yet to be elucidated.

TLR3 mRNA expression was significantly higher in embryonic tissues on 12th ED during development in the present study. On the contrary, it seems likely that low expression of *TLR3 in utero* in mammals is a developmentally desirable trend. High *TLR3* levels have been linked to a higher frequency of abortion in a mouse model as well as in human (Clark *et al.,* 2004; Zhang *et al.,* 2007). Possibly elevated fetal expression of TLR3 before birth carries with it an elevated risk of deleterious fetal–maternal interactions or of autoimmune pathology. However, this may be due to extra uterine development in avian species. Moreover, TLR3 is a negative regulator of human embryonic neural progenitor cell proliferation (Lathia *et al.,* 2008). Significantly higher levels of *TLR7* gene expression was observed in the intestine on 12th ED than other tissues investigated. *TLR7* is triggered by antiviral compounds and single-stranded RNA, and is implicated in the immune response to viruses such as influenza virus. *TLR7* gene is expressed in chicken and duck embryo fibroblasts and the expression is up regulated upon infection with H5N1 virus (Liang *et al.,* 2011). Moreover previous studies have also shown that TLR7 ligands can be successfully administered *in ovo*, highlighting their potential as adjuvants for *in ovo* administered vaccines (Rautenschlein *et al.,* 2000). *In ovo* administration of ligands at ED18 may result in the lag period until hatch (ED21), resulting in optimal protection being provided to newly hatched chicks.

Significantly higher levels of *TLR5* gene expression were observed in all embryonic tissues on 18th ED. TLR5 recognizes flagellin of Salmonella serovars and plays a role in restricting systemic infection by Salmonella (Iqbal *et al.,* 2005b). TLR15 is a recently described receptor and appears to be unique to avian species (Higgs *et al.,* 2006; Temperley *et al.,* 2008). The ligand specificity for TLR15 has not yet been conclusively determined, but there are substantial evidences that TLR15 recognizes some components of *Salmonella* spp. (Higgs *et al.,* 2006;

MacKinnon *et al.*, 2009b; Nerren *et al.*, 2009, 2010). Earlier, Higgs *et al*. (2006) demonstrated similarity in the expression pattern between *TLR2* and *TLR15* in caecum of *Salmonella* infected chicken and speculated dimerization of these two receptors during ligand recognition. However this similarity was not observed except for liver in the current study. *TLR5* and *TLR15* gene expression in embryonic tissues may have implication in resistance against *in ovo Salmonella* spp. infection.

Reinforcing the earlier report (Meade *et al.,* 2009a) the present study also showed significant up regulation of *TLR21* gene expression in liver and intestine during 15th and 18th ED. TLR21 is an unique receptor for avian and fish species (Temperley *et al.,* 2008). Neonatal chicks are responsive to CpG motifs in synthetic oligodeoxynucleotides (ODN) (Patel *et al.,* 2008) and birds are protected against *Salmonella* spp. and *Eimeria* spp. infection after administration of ODNs either *in ovo* or to newly hatched birds (He *et al.,* 2007; Taghavi *et al.,* 2008; Dalloul *et al.,* 2005). Only recently chicken TLR21 was demonstrated to act as a functional homologue to mammalian TLR9 in the recognition of CpG-ODN and bacterial genomic DNA (Brownlie *et al.,* 2009; Keestra *et al.,* 2010). However, on the contrary a recent study (Ciraci and Lamont, 2011) demonstrated that rapid response to multiple classes of CpG-ODNs requires more than one specific TLR. Several receptors including TLR15, TLR21 and TLR2 respond to CpG-ODNs in a sequence-specific manner and potential cross-talk may exist among them. Earlier findings and the present result of *TLR21* expression in developing chicken embryo suggests the competence of embryo to respond to CpG motifs which can be potentially exploited as immune adjuvants.

The present study demonstrated the differential expression of *TLR* genes during chick embryogenesis in the absence of infection. Constitutive expression of *TLR* transcripts in the developing chicken embryo in the absence of infection represent a preparatory mechanism to protect the embryos *in ovo* and the newly hatched chick against infections. Apart from the role of innate immunity TLRs may also have a role in embryo development. Indeed Drosophila Toll, the first member of the TLR family was initially characterized as a developmental protein before the establishment of their role in immunity (Hashimoto *et al.,* 1988; Kambris *et al.,* 2002). Hence there is a precedence to suggest a probable role of TLR expression in embryonic development in addition to an innate immune response.

Comparative quantification of constitutive ß-defensins mRNA expression in Indian native and White Leghorn chickens

Avian ß defensins (AvBDs) are now considered as one of the key components of innate immunity in avian species. Defensins act as a first line of defense against invading pathogens and execute anti-microbial activity by non-oxidative mechanisms (Sahl *et al.,* 2005). Some defensins are also chemoattractant for monocytes, lymphocytes and dendritic cells, thus acts as a link between innate and adaptive immune responses (Yang *et al.,* 1999; Ganz, 2003). A total of 14 ß-defensin genes (*AvBD1-14*) have been identified in the chicken (Lynn *et al.,* 2004, 2007; Xiao *et al.,* 2004). Differential constitutive expression of *AvBD* genes in developing chicken embryonic tissues as well as neonatal chicken tissues have been demonstrated earlier (Lynn *et al.,* 2004; Bar-Shira and Friedman, 2006; Ma *et al.,* 2008; Meade *et al.,* 2009a). Recently, the tissue specific expression profile of *AvBD* genes has also been studied in singing bird (zebra finch) and found to be similar to that of chicken to a great extent (Hellgren and Ekblom, 2010). Innate immune system is crucial in very young chickens as the acquired immune system fully develops only after the first week of age (Bar-Shira *et al.,* 2003). In the present study, the mRNA expression levels of all 14 *AvBD* genes in spleen tissue of day-old chicks of Aseel and Kadaknath were quantified and compared with that of White Leghorn.

Spleen has been chosen since it is one of the secondary lymphoid organs that play a major role in eliciting the immune responses against infections (Jeurissen, 1991). This differential expression of *AvBD* genes in spleen tissue of chicken breeds suggests the difference in their antimicrobial as well as other immune-modulatory functions. Moreover the difference among the breeds draws speculation towards difference in innate immune-competence.

Earlier reports together with present result of expression of *AvBD* transcripts in day-old-chicks in the absence of infection suggest the innate preparedness of younger chicken to encounter pathogens immediately post-hatch. On the contrary to the earlier findings of weak or nil expression of *AvBD2* in chicken spleen (Zhoa *et al.,* 2001; Lynn *et al.,* 2004; van Dijik *et al.,* 2008), moderate level of mRNA expression of *AvBD2* in spleen was observed in all three breeds. However this is in the agreement with the findings of other workers who reported stronger transcript expression of *AvBD2* in spleen of duck (Soman *et al.,* 2009a; Ma *et al.,* 2009a) and zebra finch (Hellgren and Ekblom, 2010). The observed discrepancy may reflect the difference in species (duck, zebra finch versus chicken) that adapt to encounter different pathogens in different habitat. AvBD1 and 2 were first purified from chicken heterophils (Evans *et al.,* 1995) and a recent report demonstrated the ability of avian intestinal epithelial cells to produce AvBD1 and AvBD2 (Derache *et al.,* 2009). Strong expression of *AvBD2* in bone marrow (van Dijik *et al.,* 2008) and strong to moderate expression in spleen, in the present and earlier studies suggest that it is a myeloid defensin and is crucial in systemic immune response apart from direct antimicrobial activity. Recently adjuvant and chemotactic property of chicken AvBD1 (Zhang *et al.,* 2010) and duck AvBD2 (Soman *et al.,* 2009b) have been demonstrated apart from their direct anti-microbial activity. *AvBD3* is constitutively expressed in the avian skin and tongue and inducible in trachea (Zhao *et al.,* 2001) and its anti-bacterial and LPS neutralizing activity have also been demonstrated (Bommineni *et al.,* 2007). Further, single nucleotide polymorphisms in *AvBD3* and *AvBD7* genes in broilers were found to be associated with antibody levels of SE vaccination (Hasenstein and Lamont, 2007). An earlier study demonstrated that AvBD4, AvBD7 and AvBD9 possess bactericidal activity against *Salmonella* spp. (Milona *et al.,* 2007). Moreover recombinant duck AvBD9 and 10 peptides have also been shown to possess antimicrobial activity against several bacterial strains (Ma *et al.,* 2009b). In contrast to earlier findings of weak or low expression of *AvBD7* in spleen (Xiao *et al.,* 2004; Lynn *et al.,* 2004), in the present study strong expression of *AvBD7* was observed in all three breeds in comparison to other AvBDs. Moderate expression of *AvBD9* and weak or low expression of *AvBD10* have been documented in chicken (Lynn *et al.,* 2004; Xiao *et al.,* 2004), duck (Ma *et al.,* 2009b), quail (Wang *et al.,* 2010) and zebra finch (Hellgren and Ekblom, 2010) spleen. Reinforcing these results moderate levels of expression was observed for both these defensins in the current investigation. Expression of transcripts of these *AvBD*s (*4, 7, 9* and *10*) in spleen suggests an innate protective mechanism against *Salmonella* infection in these chicken breeds.

Significant difference in *AvBD5* gene expression was observed among the breeds studied (Table 4.3). The gene expression was significantly higher in Kadaknath than Aseel and White Leghorn. However, no significant difference was found in the mRNA expression of

AvBD8, *9*, *10* and *11* among the breeds investigated. AvBD11, an antimicrobial peptide (AMP) with broad antimicrobial efficacy was recently found to be one of the most abundant proteins in the vitelline outer membrane of the egg (Mann, 2008) and significantly upregulated during embryogenesis (Meade *et al.,* 2009a). *AvBD11* was strongly expressed in spleen and small intestine of broiler chicks and was predominantly active against the intestinal pathogens (Higgs *et al.,* 2005). *AvBD12* was differentially expressed among these three breeds and was significantly higher in Aseel in comparison to Kadaknath and White Leghorn in the present study. AvBD13 modulates the adaptive immune responses of chickens *in vivo* and *in vitro* (Yang *et al.,* 2007). Recently a study suggested that AvBD13 maybe an endogenous ligand for TLR4 and that AvBD13 enhances the proliferation of monocytes via the NF-?B pathway (Yang *et al.,* 2010). Although significant difference in *TLR4* mRNA expression among Indian native chicken and White Leghorn chicken was found in an earlier study (Kannaki *et al.,* 2010a), in the present study we did not observe any significant difference for *AvBD13* gene expression among the native chicken and White Leghorn. Interlink between *TLR* and *AvBD* gene expression has yet to be established. *AvBD14* was recently identified member of AMP family (Lynn *et al.,* 2007) and this gene expression was significantly higher in Aseel than other two breeds in the present investigation. In summary, the present study revealed significantly higher mRNA expression levels for *AvBD* (*5*, *12* and *14*) genes in Indian native chicken breeds when compared to White Leghorn.

Differential innate immune gene expressions in the gastrointestinal tract of chicken upon *S.* Pullorum-infection

Salmonella Pullorum, a fowl-specific pathogen causes systemic salmonellosis resulting in substantial mortality in chicks (Shivaprasad, 2000; Wigley *et al.,* 2001). Persistence of infection or carrier-status and subsequent vertical transmission through eggs are the major concerns with *S*. Pullorum infections of birds more than a week old (Chappell *et al.,* 2009). Innate immune gene expressions have been studied for SE and *S.* Typhimurium infections in chicken (MacKinnon *et al.,* 2009b; Shaughnessy *et al.,* 2009). Unlike infections with SE or *Salmonella* Typhimurium, *S*. Pullorum does not induce an inflammatory response in chicken intestine (Henderson *et al.,* 1999; Kaiser *et al.,* 2000) and other studies also suggested that the pattern of immune response induced by *S*. Pullorum invasion might be distinct (Chappell *et al.,* 2009).

Differential expression pattern of TLRs in developing chicken embryonic gut tissues as well as neonatal chicks in the absence of infection status have been earlier demonstrated by several authors (Higgs *et al.,* 2006; Abasht *et al.,* 2008; MacKinnon *et al.,* 2009a; Meade *et al.,* 2009a; Kannaki *et al.,* 2011). The constitutive *TLR* expression in the gut tissues of younger birds indicates the innate preparedness to encounter pathogens that enter via feed or from the environment immediately post-hatch (Bar-Shira and Friedman, 2006). In the present study, *TLR*s and *AvBD* gene expression levels were investigated in GI tissues (duodenum, jejunum, ileum and caecum) of 3-day-old broilers after 24 h of oral infection with virulent isolate of *S*. Pullorum.

In the present study TLR1LA, TLR1LB and TLR2B gene expressions remained unchanged upon *S*. Pullorum infection. Chicken TLR1 and TLR2 gene have been duplicated, evolved independently and recently designated as TLR1LA, TLR1LB, TLR2A and TLR2B (Temperley *et al.,* 2008). These receptors are involved in the recognition of diacylated- or triacylated bacterial lipopeptides (Fukui *et al.,* 2001; Keestra *et al.,* 2007). In addition, the chicken TLR2A/TLR1LB heterodimer has been reported to be activated by peptidoglycan (Higuchi *et al.,* 2008). However, *TLR2A* gene expression was significantly up regulated in ileum of infected chicken in similarity to MacKinnon *et al.* (2009b). This increased gene expression may facilitate in increased recognition of lipoproteins and peptidoglycan. TLR4 and possibly TLR2 recognize LPS, and polymorphisms in TLR4 gene is linked with susceptibility to *Salmonella* spp. infection in chicken (Leveque *et al.,* 2003; Kogut *et al.,* 2005b). In the present study significant up regulation of *TLR4* gene was observed in duodenum and ileum of infected chicken (Fig. 4.5). MyD88-dependent pathway of TLR4 signaling has been shown to be upregulated in young chickens infected with *S*. Pullorum (Li *et al.,* 2010). Reinforcing earlier studies *TLR4* gene expression was not upregulated in the ceca after infection (Higgs *et al.,* 2006; MacKinnon *et al.,* 2009b; Li *et al.,* 2010). Moreover, high degree of similarity in gene expression was observed for *TLR2* and *TLR4*. This similarity supports the results of earlier workers suggesting the possible dimerization or cross-regulation of these two receptors (Abasht *et al.,* 2008, 2009; MacKinnon *et al.,* 2009b).

Chicken TLR5 recognizes and responds to SE flagellin (Iqbal *et al.,* 2005b; Keestra *et al.,* 2008). Downregulation of *TLR5* gene expression was earlier reported as an immune evasion mechanism to limit overstimulation by SE during infection in chickens (Abasht *et al.,* 2008; MacKinnon *et al.,* 2009a). As expected, *TLR5* gene expression was not changed in the present study upon infection with *S*. Pullorum as this serovar is an aflagellate fowl pathogen (Guard-Petter, 1997). Non-flagellated mutants of *Salmonella enterica* serovar Typhimurium demonstrated enhanced ability to systemically colonize in neonatal chicks after oral challenge suggesting chicken TLR5 may play a role in restricting systemic infection by *Salmonella* spp. (Iqbal *et al.,* 2005b). Lack of TLR5 stimulation by *S.* Pullorum may be one of the important mechanisms that facilitates in bacterial colonization and pathogenesis. Further, *TLR3* and *TLR7* gene expressions remained unchanged in all GI tissues except jejunum after *S.* Pullorum infection in this present study. This is not surprising since both TLR3 and TLR7 are receptors for viral ligands, they did not get altered by bacterial infection. Earlier, MacKinnon *et al.* (2009b) also observed similar pattern in GI tissues of SE infected chicken. However, in the present study, significant down regulation of *TLR3* gene expression in jejunum of infected chicken was observed; the reason could not be explained with the present knowledge.

TLR15 is a recently described avian specific receptor with no vertebrate ortholog counterparts (Higgs *et al.,* 2006; Temperley *et al.,* 2008). The ligand specificity for TLR15 has not yet been conclusively determined. In contrast to some earlier reports (Higgs et al., 2006; MacKinnon *et al.,* 2009a; Shaughnessy *et al.*, 2009) unaltered *TLR15* gene expression was found upon *S.* Pullorum infection in most of the GI tissues, except for jejunum in which *TLR15* gene expression was down regulated. The differences observed between these studies may be as a result of differences in bacterial strains (SE, *S.* Typhimurium versus *S.* Pullorum). Similar pattern of unchanged *TLR15* gene expression was also observed in response to *C. jejuni* infection in chicken (Shaughnessy *et al.,* 2009). However, the suggested similarity in the gene expression pattern between *TLR2* and *TLR15* (Higgs et al., 2006) was not observed in the current study. Unaltered expression and down regulation of *TLR15* gene expression suggests a similar mechanism of immune evasion found for *TLR5* gene expression during SE infection in chicken (van Aubel *et al.,* 2007; MacKinnon *et al.,* 2009). Hence, this expression pattern of *TLR15* gene in response to *S.* Pullorum infection highlights key differences among different bacterial species in the induction of immune response.

TLR21 is an unique receptor for avian and fish species. This gene expression was significantly upregulated in all of the GI tissues investigated. However, this is in contrast to the finding of MacKinnon *et al.* (2009b), wherein *TLR21* gene expression was found unaltered upon SE infection and they speculated this receptor for viral recognition. However, recently, chicken TLR21 has been demonstrated to act as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides and bacterial genomic DNA (Brownlie *et al.,* 2009; Keestra *et al.,* 2010). Further, it has also been demonstrated that avian TLR21 may exhibit broader bacterial species recognition through their CpG motifs than mammalian TLR9 (de Zoete *et al.,* 2010; Brownlie and Allan, 2011). Upregulation of chicken *TLR21* gene expression was also observed in *S.* Typhimurium and *C. jejuni* infection (Shaughnessy *et al.,* 2009). The present upregulation of *TLR21* gene expression in GI tissues may play an important role in *S.* Pullorum pathogenesis. The present study has demonstrated the upregulation of some of the chicken TLRs (TLR2, TLR4 and TLR21) upon infection with *S.* Pullorum, however, the magnitude is far less than infection with SE or *S.* Typhimurium (MacKinnon *et al.,* 2009b; Shaughnessy *et al.,* 2009). Earlier investigations also suggested that *S.* Pullorum infection causes only limited inflammation (Henderson *et al.,* 1999). Moreover, infection with this serovar downregulates inflammatory chemokine gene expression CXCLi1 in ileum during early stages of bacterial invasion and mediates a bias towards Th 2 responses in later stages facilitating in the development of carrier state (Kaiser *et al.,* 2000; Chappell *et al.,* 2009).

Avian defensins have been linked to host resistance to *Salmonella* spp. intestinal carriage (Sadeyen *et al.,* 2004, 2006). Expression analysis revealed that out of 14 *AvBD* genes, all were detectable in all GI tissues both uninfected and infected groups, while expressions of *AvBD2* and 7 were at very low level in both groups. Differential constitutive expression of AvBD genes in developing chicken embryonic tissues as well as neonatal tissues have been demonstrated earlier (Lynn *et al.,* 2004; Bar-Shira and Friedman, 2006; Ma *et al.,* 2008; Meade *et al.,* 2009a). Chicken AvBD1 and 2 were isolated from heterophils whilst chicken AvBD3-13 were shown to be constitutively expressed in the epithelia of a range of tissues

(Zhao *et al.,* 2001; Lynn *et al.,* 2004; Higgs *et al.,* 2005). Our results together with earlier reports demonstrate the ability of avian intestinal epithelial cells to produce AvBD1 and AvBD2 (Derache *et al.,* 2009). Minor discrepancies were observed in constitutive expression pattern of ß defensin genes between the present results and earlier studies. It can be speculated that these discrepancies between these studies reflect differences between the experimental conditions such as variation in breeds, age and techniques used (semi-quantitative RT-PCR vs. real-time PCR). These findings together with present results suggest the innate preparedness of younger chicken to encounter pathogens that enter via feed or from the environment immediately post-hatch. Moreover, differential expression pattern of the ß defensin family suggests their spectrum of antimicrobial and immunomodulatory activity. Present finding of low expression of *AvBD2* is in consistence with an earlier study (Lynn *et al.,* 2004), which also reported moderate to low expression of this defensin in intestinal tissues. Similar to the present result, Soman *et al.* (2009a) also reported very weak expression of *AvBD2* in duck intestine tissues suggesting the lack of its role in primary defense in the digestive tract. Further, due to its strong expression in bone marrow it is considered as myeloid defensin and is crucial in systemic immune response rather than protecting the epithelial surfaces. Recently adjuvant and chemotactive activity of chicken AvBD1 (Zhang *et al.,* 2010) and duck AvBD2 (Soman *et al.,* 2009b) have been demonstrated apart from their direct anti-microbial activity.

In the present study, quantitative real-time PCR analysis revealed significant upregulation of gene expressions of *AvBD3, 4, 5, 6* and 12 and a significant down regulation in the gene 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 (Fig. 4.6). Akbari *et al.* (2008) also reported that *AvBD1* and *2* gene expressions were not changed in caecal tonsils upon infection with *S.* Typhimurium. Recently Derache *et al.* (2009) also observed no change in *AvBD2* gene expression in intestinal epithelial cells upon *Salmonella* challenge in *in vitro* assay. However, in contrast to present results, Sadeyen *et al.* (2006) earlier reported increases in the gene expression levels of *AvBD1* and *AvBD2* in the cecal tonsils of chicken inbred lines with *Salmonella* resistant trait. The observed discrepancy may be a result of differences in bacterial strains (SE, versus *S.* Pullorum).

AvBD3 was constitutively expressed in the avian skin and tongue and inducible in trachea (Zhao *et al.,* 2001). Moreover, anti-bacterial and LPS neutralizing activity of AvBD3 have also been demonstrated (Bommineni *et al.,* 2007). AvBDs play significant roles in host resistance to *Salmonella* spp. colonization as indicated by the correlation between a high level expression of AvBDs and a low level of *Salmonella* spp. load in the caecum (Sadeyen *et al.,* 2006). Single nucleotide polymorphisms in *AvBD3* and *AvBD7* genes in broilers were found to be associated with antibody levels of SE vaccination (Hasenstein and Lamont, 2007). *AvBD3* and *12* gene expressions were upregulated in *S.* Typhimurium infection (Meade *et al.,* 2009b). Earlier study demonstrated that AvBD4, AvBD7 and AvBD9 possess bactericidal activity against Salmonella species (Milona *et al.,* 2007). In another study, the expression of AvBD6 in the cecal tonsils of 6-week-old broiler chickens and its antimicrobial activity against enteric pathogens were shown (van Dijk *et al.,* 2007). Upregulation of *AvBD4* and *AvBD6* in duodenum upon *S.* Pullorum may be important in encountering initial bacterial intestinal colonization. Earlier studies indicated that *AvBD7* gene expression to be ubiquitous while *AvBD4* and *AvBD9* gene expressions appeared localized to specific epithelial tissues including the ovary, trachea, and lung, respectively (Milona *et al.,* 2007).

In similarity to present results, Milona *et al*. (2007) earlier observed no change in the expression of *AvBD7* and *9* gene expressions in small intestine upon oral challenge with *Salmonella*. Higgs *et al.* (2005) showed the strong expression of *AvBD11* in small intestine of day-old broiler and demonstrated the antimicrobial activity of synthetic AvBD11 peptide against a range of pathogens and found to be most active against bacteria residing in the intestine. AvBD13 modulates the adaptive immune responses of chickens *in vivo* and *in vitro* (Yang *et al.,* 2007). However, in the present study *AVBD11, 12* and *13* gene expressions were significantly down regulated in GI tissues upon *S.* Pullorum infection. Similar down regulation of *AvBD3, 8, 13* and *14* gene expressions were found in *C. jejuni* infection model in chicken (Meade *et al.,* 2009b). In mouse and rat models, studies have shown that defensins could be down regulated by *Salmonella* in the intestine (Rodenburg *et al.,* 2007; Salzman *et al.,* 2003). This down regulation may be an immune evasion mechanism employed by *S.* Pullorum to avoid inflammatory reaction in initial bacterial colonization phase.

Recognition of pathogen-associated molecular pattern (PAMP) by toll-like receptors (TLR) activates nuclear factor kappa B (NF-?B) and mitogen-activated protein kinase (MAPK), leading to the up-regulation of beta defensin-2 (Vora *et al.*, 2004). Recently a study suggested that AvBD13 maybe an endogenous ligand for TLR4 and that AvBD13 enhances the proliferation of monocytes via the NF-?B pathway (Yang *et al.*, 2010). Significant upregulation of *TLR2*, *4* and *21* gene expressions in GI tract tissues of 3-day-old broiler chicks infected with S. Pullorum was also observed in the present study. It is likely that LPS, flagellin, and/or secreted virulence factors of *Salmonella* spp. function as PAMP to trigger the expression of TLRs and subsequent AvBDs by complex interlinked pathways. It appears that the ß defensin genes play a role in the transition from an innate immune response to an adaptive response in the newly hatched birds apart from having direct anti-microbial activity (Ganz, 2003; Bar-Shira and Friedman, 2006).

Identification and molecular characterization of duck and turkey *TLR* genes

Advances in whole genome sequencing and annotation in recent times have led to the identification of TLRs in several vertebrates including fish (Oshiumi *et al.*, 2003), amphibian (Ishii *et al.*, 2007), birds (Yilmaz *et al.*, 2005; Boyd *et al.*, 2007; Temperley *et al.*, 2008) and mammals (Medzhitov *et al.*, 1997). However the present knowledge of avian TLR family is solely based on chicken studies (Fukui *et al.*, 2001; Boyd *et al.*, 2001; Iqbal *et al.*, 2005b; Philbin *et al.*, 2005; Kogut *et al.*, 2005b; Higgs *et al.*, 2006; Keestra *et al.*, 2007). Recently *TLR* genes and pathway components have been annotated in zebra finch (*Taeniopygia guttata*), a passseriform bird from the genome draft (Cormican *et al.*, 2009). Despite being distantly related to the chicken, analysis of zebra finch genome showed overall conformity of TLR family and downstream signaling components (Cormican *et al.*, 2009). However, there is dearth of information regarding TLR repertoire in other avian species. Ducks are the primary hosts and natural reservoir of influenza. Avain influenza H5N1 strains, which are highly pathogenic to chickens, rarely harm ducks (Hulse-Post *et al.*, 2005). However, immunological basis for this difference is still unknown. Hence, the recently published turkey genome (Dalloul *et al.*, 2010) and duck genome (unpublished) were analyzed by *in silico* approach for identifying TLR repertoire in these two economically important avian species.

All ten *TLR* genes orthologus to chicken genes are present in both duck and turkey genome (Table 4.6 and 4.7, respectively). *TLR1* and *2* genes are duplicated in chicken, duck, turkey and zebra finch to give rise to *TLR1LA*, *TLR1LB, TLR2A* and *TLR2B* (Temperley *et al.,* 2008; Cormican *et al.,* 2009). The duplication of *TLR1* and *TLR2* genes are characteristic of avian lineage as they are found in almost all the bird species studied till today (Cormican *et al.,* 2009; Dalloul *et al.,* 2010). The avian *TLR1A/B* and *TLR2A/B* genes are orthologs of mammalian *TLR1/6/10* and *TLR2* respectively (Dalloul *et al.,* 2010). The duplicated *TLR2* genes in avian species are under positive selection to retain both the members. Both positive selection and gene conversion shape the evolution of the avian specific *TLR2* genes (Cormican *et al.,* 2009). The duplication of *TLR2* gene in avian lineage is not an unique event as other independent duplication of *TLR2* gene have occurred in American alligator (*X. tropicalis*) (Beutler and Rehli, 2002) and in the ancestor of marsupial and eutherian mammals (Roach *et al.,* 2005). However, the timing of these duplications remains unknown. Interestingly, there are remnants of second disrupted *TLR2* like gene in tandem with functional TLR2 in mice and humans. Hence, the duplication of *TLR2* gene might have occurred prior to the divergence of mammals and birds and subsequently lost its functionality in mammalian lineage. In mammals TLR2 has been shown to be the common heterodimer partner for the members of TLR1 family members (TLR1/6/10) in the recognition of diacyl and triacyl lipopeptides (Takeuchi *et al.,* 2001). Recently it has been demonstrated that chicken TLR1Ls interact with TLR2s and can recognize agonists identical to those of mammalian heterodimers (Fukui *et al.,* 2001; Keestra *et al.,* 2007; Higuchi *et al.,* 2008). However the functional characterization of these TLR receptors is yet to be done in other avian species.

Aves appear to miss a number of TLRs which are present in most of mammals. *TLR7*, *8* and *9* subfamily is represented only by *TLR7* gene in chicken, duck, turkey and zebra finch (Temperley *et al.,* 2008; MacDonald *et al.,* 2008: Cormican *et al.,* 2009) nevertheless *TLR7* is duplicated in the later. *TLR8* gene in birds has been disrupted; whereas *TLR9* gene has been deleted in the course of evolution (Philbin *et al.,* 2005; Temperley *et al.,* 2008). Philbin *et al.* (2005) demonstrated that *TLR8* gene is disrupted by a retroviral, CR1-type insertion element only in galliform species and not in anseriform birds including ducks. However, later it was disproved and duck genome was shown to have disrupted *TLR8* gene (Roach *et al.,* 2005: MacDonald *et al.,* 2008). *TLR7* and *TLR8* genes are adjacent to each other in the genomes of fish and mammals (MacDonald *et al.,* 2008). Hence genomic region downstream to TLR7 region was examined in this study and identified CR1 like elements in between fragments having homology to mammalian *TLR8* gene in duck and turkey genome. Complicated overlap of function between the TLR7/8 in mouse and human has led to the speculation that mouse *TLR8* and human *TLR7* are evolving to become pseudogenes (Crozat and Beutler, 2004). Indeed, the loss of *TLR8* in avian lineage reflects similar evolutionary pressure.

Similar to chicken no mammalian ortholog of *TLR9* and *10* genes could be found in duck and turkey genome. In spite of *TLR9* gene deletion in chicken genome oligodeoxynucleotides (ODN), a synthetic TLR9 agonist have been shown to be effective in chicken against bacterial infections (Gomis *et al.,* 2003, 2004; He *et al.,* 2007; Taghavi *et al.,* 2008). Chicken possess TLR21 orthologous to fugu and Xenopus TLR21 (Temperley *et al.,* 2008). TLR21 was also found in duck and turkey genome in the present analysis. Recently it has been demonstrated that chicken TLR21 but not TLR7 or TLR15 as speculated earlier recognizes unmethylated CpG motifs and acts as functional homolog to mammalian TLR9 (Brownlie *et al.,* 2009; Keestra *et al.,* 2010). Further chicken TLR21 exhibits broader ligand specificity compared with mammalian TLR9 as apparent from the response to both human and murine specific TLR9 ligand as well as bacterial genomic DNA (Brownlie *et al.,* 2009; Keestra *et al.,* 2010). Comparative sequence analysis of chicken, duck, turkey and human TLR9 revealed that similar to chicken TLR21 (Keestra *et al.,* 2010), duck and turkey TLR21 also lack insertion sequence between LRR15 and LRR16 typical for human TLR7-9, involved in proteolytic cleavage and implicated in TLR9 like function (Latz *et al.,* 2007; Ewald *et al.,* 2008; Peter *et al.,* 2009). Further analysis of ectodomain LRR motifs revealed that avian TLR21 lacks the double CxxC motif that is present in LRR8 of murine and human TLR7-9 (Bauer and Wagner, 2002). These structural differences suggest that avian TLR21 employ yet another mechanism for DNA binding and receptor activation and may have evolved convergently to recognize the same ligand. The presence of TLR9 in mammals, TLR21 in chicken and both receptors in fish imply that avian species may have lost during the course of evolution probably needlessness to distinguish different DNA motifs.

The expression pattern and distribution of the TLRs have been shown to be characteristic of each species (Zarember and Godowski, 2002; Kogut *et al.,* 2005a; Firth *et al.,* 2005; Nalubamba *et al.,* 2008; Vahanan *et al.,* 2008; Tirumurugaan *et al.,* 2010). Expression pattern of TLR genes have been studied for chicken, duck (*TLR7*) and zebra finch (*TLR4*) (Kogut *et al.,* 2005a; Iqbal *et al.,* 2005a; Higgs *et al.,* 2006; MacDonald *et al.,* 2008; Vinkler *et al.,* 2009). The present study profiled the expression of *TLRs* mRNA in various tissues of ducklings and turkey poults and also comparatively quantified their transcript expression levels by real-time PCR assay. Measurable mRNA expression for each TLR in a range of tissues from both duck and turkey poults was observed. In similarity to earlier finding in duck (MacDonald *et al.,* 2008), *TLR7* was highly expressed in spleen and bursa. In addition, high expression is also observed in the lung tissue of duck, which is distinct from the expression pattern of chickens (Kogut *et al.,* 2005a; Iqbal *et al.,* 2005a). The observed difference in *TLR7* expression may be due to differences in organization of lymphoid tissue in the lung of ducks and chickens, or the presence of resident cells expressing the TLR7 receptor, which are absent in the chicken. The duck *TLR7* expression pattern is comparable to that for humans, which is highest in spleen, with significant expression in lung (Nishimura and Naito, 2005). High pulmonary expression of *TLR7* gene could be significant in the context of highly pathogenic H5N1 avian influenza, which is primarily a lung infection. Further, differences were found in the expression levels of transcripts of different TLRs between duck, turkey and other bird species. This may be an adaptation to encounter different range of pathogens specific to each species. He *et al.* (2008) showed differential induction of nitric oxide in response to microbial agonist stimulations in monocytes and heterophils from young commercial turkeys. This finding together with our results of *TLR* gene expression in young turkey poults demonstrates the innate preparedness of young birds to encounter pathogens. The greater initial *TLR* gene expression in younger birds indicates the potential for a stronger innate immune response.

Molecular characterization of TLR15 of avian species

TLR15 is considered as specific receptor to avian, more specifically to chicken as homologues of TLR15 have not yet been found in searches of available vertebrate genome (Higgs *et al.,* 2006). However, as chicken is the only completely sequenced avian genome, this does not preclude the existence of TLR15 in other avian species. At least TLR15 must exist in avian species if not in other vertebrates. To confirm this speculation, a recent bioinformatic analysis of recently submitted zebra finch (*Taeniopygia guttata*) genome, one of the three currently available avian genome revealed the presence of *TLR15* gene (Cormican *et al.,* 2009). Presence of *TLR15* in zebra finch, a passeriform species, it is evident that TLR15 must have evolved well before the galliformes-passeriformes split (G-P split). Hence, TLR15 must be conserved among the avian lineage.

Chicken TLR15 is molecularly distinct from all known TLRs (Roach *et al.,* 2005; Higgs *et al.*, 2006). The present study confirmed the presence of TLR15 in turkey, quail and indigenous chicken breeds. Analysis of domain structure of TLR15 revealed an archetypal TLR structure comprising of extracellular ligand binding domain including variable number of leucine-rich repeats (LRRs) and the cytoplasmic toll/interleukin-1 (IL-1) receptor (TIR) domain involved in signaling. The length of ectodomain, transmembrane domain and TIR domain was found to be conserved in both chicken breeds, turkey and quail, although minor differences were found in number of LRRs and their position. Number of LRRs and their phasing vary among TLR family members (Matsushima *et al.,* 2007; Temperley *et al.,* 2008). Indigenous chicken breeds possess 10 LRRs in their ectodomain region similar to chicken sequence, whereas Japanese quail sequence revealed 11 LRRs, one additional LRR at C-terminal and slightly vary in LRRs positions from commercial chicken sequence. In similarity to chicken sequence these LRRs are clustered towards C-terminus of the molecule (Temperley *et al.,* 2008). In contrast to other vertebrate TLRs, there are no N-terminal cysteine clusters in TLR15, which forms a cap like structure to shield the hydrophobic core of first LRR. Interestingly, this feature is shared by vertebrate TLR1 family (TLR1, TLR2, TLR6 and TLR10) too (Matsushima *et al.,* 2007). Furthermore, chicken TLR15 is coded by a single exon, a feature common to all mammalian members of TLR1/2/6/10 clade. Comparing the ectodomain of vertebrate TLR7 family (TLR7, TLR8 and TLR9) to chicken TLR15 revealed some similarity. Chicken TLR15 possess two horseshoe domains of LRRs, a feature observed only in vertebrate TLR7 family. Moreover, LRR of TLR7 family and TLR15 forms deeper arc rather than flat arc as that of other TLRs (Matsushima *et al.,* 2007). However the significance of these common features in ligand recognition cannot be predicted at this stage.

Phylogenetic analysis revealed TLR15 grouped with TLR1 family with high bootstrap support reinforcing the results of earlier studies (Higgs *et al.,* 2006; Temperley *et al.,* 2008) (Fig. 4.15). Avian TLR1 family is represented by a single member unlike many members (TLR1/6/10) of mammalian TLR1 family. From this it can be speculated that TLR15 compensate the lack of variability of avian TLR1 family. The ligand specificity for TLR15 has not yet been conclusively determined, but there are substantial evidences that TLR15 recognize unique, non-secreted, heat-stable component of both Gram (+) and Gram (-) bacteria of avian specific pathogens more specifically some components of *Salmonella* spp. (Higgs *et al.,* 2006; MacKinnon *et al.,* 2009b; Nerren *et al.,* 2009, 2010). Transcripts of *TLR15* increased in tissues of *S. enterica* serovar Typhimurium infected birds (Higgs *et al.,* 2006). Moreover, *TLR15* mRNA expression was significantly higher in caecum of SE infected birds (MacKinnon *et al.*, 2009b). The basal levels of mRNA expression of *TLR15* were greater in heterophils from *Salmonella*- resistant chickens than in heterophils from *Salmonella*- susceptible chickens (Nerren *et al.,* 2009). There is also some speculation that TLR15 may form heterodimer with TLR2 as gene expression pattern of *TLR2* and *TLR15* mRNA was highly similar in Salmonella-infected chicken (Higgs *et al.*, 2006; Mackinnon *et al.,* 2009b). Dimerization of vertebrate TLR1 family members with TLR2 during the course of ligand recognition is well established (Keestra *et al.*, 2007; Higuchi *et al.*, 2008). Although collectively these studies strongly suggest that TLR15 plays a role in immune response to multiple serovars of *Salmonellae*, in a recent study *in vitro* stimulation of chicken heterophils with purified TLR agonist, heat killed and formalin inactivated whole bacteria demonstrated that individual TLR agonists are not the ligand for TLR15 and it recognizes unique, non-secreted, heat-stable component of both Gram (+) and Gram (-) bacteria of avian specific pathogens (Nerren *et al.,* 2010). Further, TLR15 may form heterodimer with TLR2 similar to that of TLR1 members as gene expression pattern of both was highly similar in *Salmonella*-infected chicken (Higgs *et al.,* 2006; Mackinnon *et al.,* 2009b). Therefore TLR15 in avian lineage may have evolved as novel heterodimeric partner for TLR2. Proteins with conserved functions may undergo purifying selection to eliminate deleterious mutations (Medzhitov, 2001). Analysis of known avian TLR sequences in this study revealed that they were under purifying selection.

Functional characterization of duck TLRs

In vitro immuno stimulation of duck PBMC cultures with TLR agonists LPS and poly I:C resulted in significant ($P<0.05$) upregultion of *IL-1ß* cytokine gene expression in LPS stimulated culture after 12 and 24 h. Whereas no significant difference in gene expression of IFN-? was observed in poly I:C induced culture. Similar to chicken (He *et al.*, 2006), duck PBMCs are also capable of producing *IL-1ß* upon LPS stimulation. These findings are consistent with the fact that receptor activation of polymorphonuclear cells induces transcriptional signals that subsequently mediate host responses. The present results are also similar to the study of MacDonald *et al.* (2008), in which only slight upregulation of interferon gene expressions were observed upon TLR agonists stimulation of duck PBMCs and splenocytes. However this is in contrast to IFN-? stimulation by poly I:C in chicken primary macrophage culture (He *et al.*, 2007). Differential activation of cytokine gene expression upon TLR agonist's stimulation was earlier reported in different chicken genetic lines (Kogut *et al.*, 2005b). Hence, the observation in present study may be due to variation in genetic background and needs to be investigated further.

Cloning, expression and purification of recombinant chicken AvBD2

In the present study, chicken AvBD2 mature peptide encoding gene region was cloned and expressed in *E. coli* system. Antimicrobial potency has been shown previously not to be significantly altered by the presence of a His-tag (Yenugu *et al.*, 2003). However, expression of the anti-microbial proteins in recombinant form in prokaryotic system can be difficult due to their toxicity to the bacterial cells (Piers *et al.*, 1993). Toxic effects of chicken AvBD2 were not observed in this study. This may be because the protein formed insoluble inclusion bodies during recombinant expression. The level of expression of the mature protein, with an N-terminal His tag, was considerably low. Earlier, Soman *et al.* (2009a) reported that addition of a part of signal peptide to duck AvBD2 mature peptide increased the level of expression of recombinant protein in *E. coli* system. However, in the present study, that step was not attempted.

Previous reports on antimicrobial activity of avian ß-defensins indicate that they are active against both Gram-positive and Gram negative bacteria (Sugiarto and Yu, 2004; van

Dijk *et al.*, 2008). The Gga_AvBD2 is active against *E. coli* and exerts the antimicrobial effect at peptide concentrations of 16 µg/ml (Evans *et al.*, 1995). Further, it is bactericidal against Gram (+) pathogen *L. monocytogenes*, however it is not effective against *Candida albicans* (Evan *et al.*, 1994; Harwig *et al.*, 1994). The Apl_AvBD2 inhibits the growth of *E. coli* at a concentration of 25 µg/ml (3.7 µM). However, the Mga_AvBD2, which has a very high amino acid similarity with Apl_AvBD2 (83.3% of the mature peptide region), did not kill *E. coli* (Evans *et al.*, 1994). Predicted amino acid sequence analysis indicated that the major variations in the two proteins lie in the N-terminal region, which forms the first ß-sheet in the secondary structure prediction. The recombinant Apl_AvBD2 also demonstrated potent antibacterial activity against Gram-positive bacteria *M. luteus* at the same concentration as that was for *E. coli*. Avian ß-defensin molecules are subjected to adaptive evolution in nature in synchrony with the host-specific pathogens (Maxwell *et al.*, 2003; Semple *et al.*, 2006; Higgs *et al.*, 2007). In the present study, the recombinant Gga_AvBD2 was found to be effective against *S.* Pullorum at a concentration of 35 µg/ml. The higher bactericidal concentration observed in this study as against reported against *E. coli* may be due to the variation in bacterial strains or secondary structure of recombinant peptide. Antimicrobial peptides must first be attracted to bacterial surfaces and the obvious mechanism is through electrostatic bonding between the peptides and structures on the bacterial surface. *Salmonella* spp. is known to resist cationic antimicrobial peptide activity by reducing the negative charge of their outer membranes through modifications of the anionic membrane molecules including LPS, with positively charged substituents (Peschel, 2002). Earlier study (Milona *et al.*, 2007) showed that recombinant AvBD9 is potent against *Salmonella* spp. than AvBD4 and 7 and attributed this to reduced charge (+4) of AvBD9. This may be one of the reasons for higher concentration required against *S*. Pullorum as AvBD2 has significantly higher charge (+9.2). This higher cationic charge may favour killing other microbes. Further experiments are required to evaluate the antimicrobial activity against range of microbes.

Salt sensitivity is one of the key features of ß-defensins, and many of these peptides are inactivated at physiological concentration of sodium chloride (~150 mM or 300 mOsm) (Tomita *et al.*, 2000). In this study, bacteria were treated with the Gga_AvBD2 in PBS, which had a sodium chloride concentration of 136 mM, and the protein exhibited antibacterial activity against *S*. Pullorum. This indicates that the chicken AvBD2 is fairly salt resistant, though the resistance cannot be compared with that exhibited by the Apa_AvBD103b at 160 mM (348 mOsm) (Thouzeau *et al.*, 2003). Information on the salt sensitivity of Gga_AvBD2 and closely related Mga_AvBD2, and Apl_AvBD2 is currently not available. Among the chicken ß-defensins previously studied, only AvBD9 has found to be relatively salt resistant at 150 mM concentration of sodium chloride (van Dijk *et al.*, 2007).

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6 Summary and Conclusions

Indigenous chicken breeds are considered to be more disease resistant than their commercial counterparts and are genetically distinct, however the immunological basis underlying this disease resistance is yet unknown. Toll-like receptor (TLR) and ß-defensins are one of the important members of evolutionarily conserved innate immune system, which is now considered as potent activator and mediator of the adaptive immune response. Constitutive expression levels of *TLR* (*2A*, *3*, *4*, *5*, *7*, *15* and *21*) genes in different tissues of day-old chicks of Aseel, Kadaknath and White Leghorn were quantified by real-time PCR. No significant difference was observed in *TLR3* gene expression among the breeds (P=0.75), however there was significant difference in gene expression among the tissue type (P=0.01). Among the tissue type lymphoid organs spleen and bursa and non-lymphoid organ heart, expressed significantly higher levels of *TLR3* transcripts followed by intestine, BM, liver and muscle in all three breeds. Significant (P=0.001) difference was found among breeds in *TLR4* gene expression. Aseel and White Leghorn expressed higher levels of *TLR4* gene expression than Kadaknath in different tissues investigated. The interaction effect between tissue type and breed was significant for TLRs (*TLR5*, *7*, *15* and *21*) gene expressions (P<0.01).

Constitutive expression levels of TLR (*2A*, *3*, *4*, *5*, *7*, *15* and *21*) genes in different tissues of developing chicken embryo were investigated. All seven *TLR* genes were expressed in whole embryonic tissue as early as 3rd embryonic day (ED). *TLR* genes are differentially expressed during chicken embryo development and each *TLR* gene showed significant changes in expression suggestive of developmental regulation. Avian ß defensins (AvBDs) are now considered as one of the key components of innate immunity in avian species. Defensins act as a first line of defense against invading pathogens and execute anti-microbial activity by non-oxidative mechanisms. The mRNA expression levels of all 14 *AvBD*s in spleen tissue of day-old chicks of Aseel and Kadaknath were quantified and compared with that of White Leghorn. Among the *AvBD* genes investigated in chicken spleen, *AvBD7* was the most expressed and *AvBD1* was the least expressed in both native chickens and White Leghorn. *AvBD5, 12* and *14* genes were differentially expressed among these three breeds. *AvBD5* gene expression was significantly higher in Kadaknath, whereas, *AvBD12* and *14* gene expressions were significantly higher in Aseel in comparison to Kadaknath and White Leghorn. These higher levels of defensin gene expressions in native chickens confer more innate immunity to them to encounter pathogens more efficiently.

*TLR*s and *AvBD* gene expression levels were investigated in gastrointestinal (GI) tissues (duodenum, jejunum, ileum and caecum) of 3-day-old broilers after 24 h of oral infection with virulent isolate of *S.* Pullorum. *TLR2A*, *TLR4* and *TLR21* gene expressions were significantly ($P<0.01$) upregulated in proximal GI segments, whereas *TLR3* and *TLR15* expressions were significantly ($P<0.05$) down regulated in jejunum after infection in *S.* Pullorum. Most substantial change in gene expression was observed for *TLR21*, being upregulated significantly ($P<0.01$) in all the GI tissues investigated. Quantitative real-time PCR analysis revealed significant upregulation ($P<0.05$) of gene expressions of *AvBD3, 4, 5, 6* and 12 and a significant ($P<0.05$) down regulation in the gene 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.

The present knowledge of avian TLR family is solely based on chicken studies. TLR repertoires in duck and turkey genome were annotated and characterized. All ten chicken *TLR* gene (*TLR1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15* and *21*) orthologs were found in both duck and turkey genome. Duck and turkey *TLR* genes showed 77 to 92% similarity at amino acid level to their chicken counter parts. Evolutionary analysis revealed the duplicated *TLR2* genes of aves are under positive selection. Expression of TLR genes were analyzed in a range of tissues of day-old ducklings and turkey poults by real-time PCR. All ten TLRs mRNA expressions were significantly higher in bursa of ducklings than other tissues studied, whereas in muscle all TLRs mRNA expressions were significantly (P<0.01) lower except for *TLR15* in duckling's tissues. *TLR7* gene expression was significantly (P<0.01) higher in spleen, bursa and also in lung of ducklings. In spleen, *TLR5* was least expressed, whereas in bursa *TLR3* was least expressed among all other TLRs investigated. Expression analysis of turkey TLR genes in a range of day-old turkey poults tissues revealed that overall TLR expression was highest for *TLR4* and lowest for *TLR21*. Turkey *TLR1A, 2A, 2B* and *21* gene expressions were significantly (P<0.01) higher in liver than other tissues investigated. *TLR3* gene expression was significantly (P<0.01) higher in marrow (BM) and spleen of turkey poults in comparison to other tissue samples.

Full length coding regions of TLR15 of Aseel, Kadaknath, J. quail and turkey were amplified, sequenced and analyzed. All four coding sequences had same ORF length of 2,607 bp encoding 868 amino acids similar to that of broiler chicken. Clustal (W) analysis revealed that Aseel, Kadaknath, Japanese quail, and turkey TLR15 sequences had 99.7%, 99.8%, 95.1%, and 92.4%, similarity with its broiler counterpart at nucleotide level respectively. Phylogentic analysis of avian TLR15 sequences showed that these sequences group with chicken TLR1 family with high bootstrap support.

In vitro immuno stimulation of duck PBMC cultures with TLR agonists LPS and poly I:C resulted in significant (P<0.05) upregultion of IL-1ß cytokine gene expression in LPS stimulated culture after 12 and 24 h. Whereas no significant difference in gene expression of IFN-? was observed in poly I:C induced culture.

Mature peptide region of chicken AvBD2 encoded by exon 3 was amplified and cloned in pUC29 cloning vector. The insert was subcloned in pET28a expression vector and induced in *E. coli* system. The 7.7 kDa recombinant peptide was expressed upon induction and purified using Ni-NTA affinity column. Anti-microbial activity of this recombinant peptide against virulent isolate of *S.* Pullorum was analyzed by microtitre broth dilution method. The minimum bactericidal concentration (MBC) of recombinant AvBD2 was found to be 35 µg/ml.

Conclusions

1. Aseel and White Leghorn expressed higher levels of *TLR4* gene expression than Kadaknath in different tissues investigated. Lymphoid organs spleen and bursa and non-lymphoid organ heart, expressed significantly higher *TLR3* gene than other tissues of all three breeds.
2. *TLR* (*2A, 3, 4, 5, 7, 15* and *21*) genes were expressed in whole chicken embryonic tissue as early as 3^{d} embryonic day (ED) and differentially expressed during development.
3. Spleen tissue of day-old-Aseel expressed significantly higher levels of *AvBD12* and *14* transcripts whereas Kadaknath had higher levels of *AvBD5* gene expression in comparison to White Leghorn.
4. Innate immune genes *TLR2A*, *TLR4*, *TLR21*, and *AvBD3*, *4*, *5*, *6* and *12* were significantly upregulated in GI tissues of 3-day-old broiler chicken after 24 h of oral infection with *S*. Pullorum. Whereas *TLR3, 15* and *AvBD10*, *11*, *13* and *14* were down regulated in upon infection.
5. All ten chicken *TLR* gene (*TLR1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15* and *21*) orthologs were found in both duck and turkey genome by *in silico* analysis. Duck and Turkey *TLR* genes showed 77 to 92% similarity at amino acid level to their chicken counter parts.
6. All ten *TLR*s mRNA expressions were significantly higher in bursa of ducklings than other tissues studied, whereas in muscle all *TLR*s mRNA expressions were significantly (P<0.01) lower except for *TLR15* in duckling's tissues.
7. Overall *TLR* expression was highest for *TLR4* in a range of day-old turkey poults tissues and lowest for *TLR21*.
8. Full length coding regions of TLR15 of Aseel, Kadanath, J. quail and turkey were sequenced and they showed 92-99.7% similarity with the broiler counterpart at nucleic acid level.
9. Mature peptide region of chicken AvBD2 was cloned and 7.7 kDa recombinant peptide was expressed in *E. coli* system.
10. The minimum bactericidal concentration (MBC) of recombinant chicken AvBD2 against virulent *S.* Pullorum was 35 µg/ ml.

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7 Mini Abstract

Toll-like receptor (TLR) and ß-defensins are one of the important members of evolutionarily conserved innate immune system which is considered as potent activator and mediator of the adaptive immune response. Constitutive expression levels of *TLR* (*2A, 3, 4, 5, 7, 15* and *21*) genes in different tissues of day-old chicks of native chicken breeds (Aseel and Kadaknath) and White Leghorn were quantified by real-time PCR. Aseel and White Leghorn expressed higher levels of *TLR4* transcripts than Kadaknath in different tissues investigated. Lymphoid organs spleen and bursa and non-lymphoid organ heart, expressed significantly higher *TLR3* gene than other tissues of all three breeds. Constitutive expression levels of TLR (*2A, 3, 4, 5, 7, 15* and *21*) genes in different tissues of developing chicken embryo were investigated. All seven *TLR* genes were expressed in whole chicken embryonic tissue as early as 3rd embryonic day (ED) and differentially expressed during development. Expression analysis of *AvBD* (1-14) genes in spleen tissue of Aseel, Kadaknath and White Leghorn revealed that Aseel expressed significantly higher levels of *AvBD12* and *14* transcripts whereas Kadaknath had higher levels of *AvBD5* gene expression in comparison to White Leghorn. Innate immune genes *TLR2A, TLR4, TLR21,* and *AvBD3, 4, 5, 6* and *12* were significantly upregulated in GI tissues of 3-day-old broiler chicken after 24 h of oral infection with *S*. Pullorum. Whereas *TLR3, 15* and *AvBD10, 11, 13* and *14* were down regulated upon infection. All ten chicken *TLR* gene (*TLR1LA, 1LB, 2A, 2B, 3, 4, 5, 7, 15* and *21*) orthologs were found in both duck and turkey genome by *in silico* analysis. Duck and Turkey *TLR* genes showed 77 to 92% similarity at amino acid level to their chicken counter parts. All ten *TLR*s mRNA expressions were significantly higher in bursa of ducklings than other tissues studied, whereas in muscle all *TLR*s mRNA expressions were significantly (P<0.01) lower except for *TLR15* in duckling tissues. Overall TLR expression was highest for *TLR4* in a range of day-old turkey poults tissues and lowest for *TLR21*. Full length coding regions of TLR15 of Aseel, Kadanath, J. quail and turkey were sequenced and they showed 92-99.7% similarity with the broiler counterpart at nucleic acid level. Mature peptide region of chicken AvBD2 was cloned and 7.7 kDa recombinant peptide was expressed in *E. coli* system. The minimum bactericidal concentration (MBC) of recombinant chicken AvBD2 against virulent *S*. Pullorum was 35 µg/ ml.

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लघु सारांश

टालसदृशग्राही (टीएलआर) एवं बीटा–डिफेन्सीन्स एक विकासमूलक संरक्षित सहज प्रतिरक्षा प्रणाली का अहम सदस्य है, जिसे अनुकूली प्रतिरक्षा प्रणाली का शक्तिशाली सक्रियक एवं माध्यस्त माना गया है। एक दिन के देशी नस्ल (असील एवं कड़कनाथ) एवं श्वेत लेगहार्न चूजों के विभिन्न उतकों में टीएलआर (2ए, 3,4,5,7,15 एवं 21) के रचनात्मक अभिव्यक्ति स्तर को वास्तविक काल पीसीआर द्वारा प्रमात्रीकरण किया गया। विभिन्न उतकों में अन्वेषण से यह पाया गया कि कड़कनाथ की अपेक्षा असील एवं श्वेत लेगहार्न चुजों में टीएलआर–4 प्रतिलेख की अभिव्यक्ति का स्तर ज्यादा था। तीनों नस्लों में अन्य सभी उतकों की अपेक्षा लसीकाभ अवयव जैसे तिल्ली एवं वसा तथा अलसीकाभ अवयव दिल में टीएलआर–3 जीन की अभिव्यक्ति सार्थक रूप से जयादा पायी गई। विकासशील कुक्कुट के भ्रूण के विभिन्न उतकों में टीएलआर (2ए, 3,4,5,6,15 एवं 21) जीन के रचनात्मक अभिव्यक्ति स्तर का अन्वेषण किया गया। सभी 7 टीएलआर जीन की अभिव्यक्ति 3 दिन के विकाशशील चूजों के भ्रूण में पायी गई, जो भ्रूण के विकास के दौरान भिन्न रूप में अभिव्यक्त हुए। AvBD (1-14) जीन के तिल्ली उतक में अभिव्यक्ति विश्लेषण से यह पाया गया कि असील चूजों में *AVBD12* एवं 14 प्रतिलेखों का अभिव्यक्ति स्तर सार्थक रूप से ज्यादा था जबकि कड़कनाथ में *AvBD5* जीन का अभिव्यंजन श्वेत लेगहार्न की तुलना में ज्यादा था।

तीन दिन के ब्रायलर चूजों के आँत्र उतकों में साल्मोनेल्ला पुलोरम से मुख अन्तःक्रमण के 24 घंटे के उपरान्त, सहजप्रतिरक्षा जीन्स टीएलआर 2ए, 4,21 एवं *AvBD* 3,4,5,6 एवं 12 का सार्थक रूप से उच्चनियमन देखा गया। इसके विपरीत संक्रमण के पश्चात टीएलआर–3, 15 एवं *AvBD 10, 11, 13, 14* जीनों का अधोनियमन पाया गया। बत्तख एवं टर्की जिनोमों के सिलिको विश्लेषण द्वारा दोनों में कुक्कुटों के दस टीएलआर जीन्स (टीएलआर–1एलए, 1एलबी, 2ए, 2बी, 3,4,5,6,7,15 एवं 21) के आरथोलाग्स पाये गये। बत्तख एवं टर्की के टीएलआर जीन्स में उनके कुक्कुट प्रतिरूपों से अमिनों अम्ल स्तर पर 77 से 92 प्रतिशत समानता पायी गई। अन्य सभी उतकों की अपेक्षा बत्तख चूजों के बरसा में सभी दस टीएलआर के दूत–आरएनए का अभिव्यंजन सार्थक रूप से ज्यादा था, जबकि टीएलआर15 को छोड़कर अन्य सभी जीन्स का अभिव्यंजन माँस उतक में कम रहा। एक दिन के टर्की चूजों के उतकों में टीएलआर–4 का अभिव्यंजन उच्चतम था एवं टीएलआर–21 का अभिव्यंजन निम्नतम था। असील कड़कनाथ, जापानीकोपल एवं टर्की के टीएलआर–15 जीन के पूरी लम्बाई कोडिंग क्षेत्रों की क्रमबद्ध किया गया एवं इनकी समानता अपने ब्रायलर प्रतिरूपों के साथ्ज्ञ नाभकिय अम्ल स्तर पर 99–99.7% थी। कुक्कुट के परिपकव पेप्टाइड क्षेत्र *AvBD-2* का ई. कोलाई प्रणाली में क्लोनीकरण किया गया एवं इसमें 7.7 केडीए पुनः संयोजित पेप्टाइड का अभिव्यंगन पाया गया। साल्मोनेल्ला पुलोरम पुनः संयोजित कुक्कुट *AVBD-2* पेप्टाइड का न्यूनतम जीवाणु मारक सांद्रण 35 μg/ml पाया गया।

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Appendix

(A) General Buffers and Reagents

1. Phosphate buffered saline (PBS pH 7.4)

Sodium Chloride	8.00 gm
Potassium dihydrogen phosphate	0.20 gm
Disodium Hydrogen Phosphate	1.16 gm
Potassium Chloride (KCl)	0.20 gm
Distilled water	upto 1000 ml

PBS was autoclaved at 121°C, 15lb pressure for 20 min and stored at 4°C for further use.

2. 1M Tris-HCl (pH 7.4 and 8.0)

Tris base (Sigma, USA)	121.1 gm
Distilled water	1000 ml

pH was adjusted to 7.4 or 8.0 as needed with 1N HCl.

3. Ethylene diamine tetra acetate (EDTA) (0.5M, pH 8.0)

EDTA	18.61 gm
Distilled water	80.0 ml

The powder was vigorously stirred while adjusting the pH with solid NaOH to 8.0 and the volume was made to 100 ml with distilled water. Autoclaved and stored at 4°C.

4. Tris-EDTA (TE buffer, pH 8.0)

Tris-HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 Mm

5. Ethidium bromide

Ethidium bromide	10 mg
Distilled water	1 ml

6. Loading dye (6X)

Xylene cyanol	0.05%
Bromophenol blue	0.09%
Glycerol	40%
EDTA (0.5M, pH 8.0)	60 Mm

The dye was autoclaved at liquid cycle and stored at 4°C.

(B) Buffers and reagents for SDS-PAGE

1. Resolving gel (10%) (for 5 ml)

Distilled water	2.0 ml
30% acrylamide monomer (Promega, USA)	1.66 ml
1.5 mM Tris HCl (pH 8.8)	1.25 ml
10% SDS (Promega, USA)	50 µl
10% Ammonium Persulfate	50 µl
TEMED	5 µl

2. Stacking gel (5%) (for 3.3 ml)

Distilled water	2.0 ml
30% acrylamide monomer (Promega, USA)	440 µl
0.5 mM Tris HCl (pH 6.8)	830 µl
10% SDS (Promega, USA)	33 µl
10% Ammonium Persulfate	20 µl
TEMED	5 µl

3. Electrode Buffer

Tris base	25 mM
Glycine	192 mM
SDS	0.1%

4. 2X Laemmli sample buffer

SDS	4%
Glycerol	20%
ß-Mercaptoethanol	10%
Bromophenol blue	0.04%
Tris-HCl (pH 6.8)	625 Mm

5. Staining solution

Coomassie Brilliant blue (R-250)	0.25%
Glacial acetic acid	10%
Methanol	45%

The solution was filtered through Watman no.1 filter paper and stored in amber coloured bottle away from sunlight.

6. Destaining solution

Glacial acetic acid	7%
Methanol	40%

(C) Buffers and reagents for bacteria culture

1. Luria Bertani (LB) medium (1X)

Bacto-tryptone	1.0 gm
Sodium Chloride	1.0 gm
Yeast extract	0.5 gm
Distilled water	90 ml

pH was adjusted to 7.5 by 1N NaOH before making the volume to 100 ml with distilled water and sterilized by autoclaving. LB agar medium was prepared by adding 1.5% agar to LB medium.

(D) Reagents for cloning and expression in *E. coli* system

1. IPTG (100mM)

IPTG	23.83 mg
Distilled water	1.00 ml

Sterilized by filtration and stored at -20$^?$C.

2. Buffers for plasmid isolation

(a) Resuspension buffer

Tris-HCl (pH 8.0)	50 Mm
EDTA (pH 8.0)	10 Mm
RNase A	100 µg/ml

Stored at 4°C.

(b) Lysis buffer

NaOH	200 mM
SDS	1%

Stored at room temperature.

(c) Neutralization buffer

Potassium acetate	3M

pH was adjusted to 5.5 by adding glacial acetic acid.

3. Recombinant protein purification buffer

(a) 1X Laemmli buffer

Tris HCl (pH 6.8)	50mM
Glycerol	10%
SDS	2%
Bromophenol blue	0.1%
DTT	100 mM

(b) Sonication buffer (buffer A)

Sodium dihydrogen phosphate	100 mM
Tris HCl (pH 8.0)	10 Mm
Guanidine hydrochloride	6 M
ß- Mercaptoethanol	230 mM
Imidazole	10 Mm
Sodium Chloride	1 M
Triton X-100	0.1%

The pH was adjusted to 8.0 with 10N NaOH.

(c) Wash buffer I (buffer B) (pH 8.0)

Sodium dihydrogen phosphate	100 Mm
Tris HCl (pH 8.0)	10 mM
Urea	8 M
ß- Mercaptoethanol	20 mM
Triton X-100	0.1%
Imidazole	10 mM
Sodium Chloride	1M

pH was adjusted with 10 N NaOH.

(d) Wash buffer II (buffer C) (pH 6.3)

Sodium dihydrogen phosphate	100 mM
Tris HCl (pH 8.0)	10 mM
Urea	8 M
Triton X-100	0.1%
Imidazole	20 mM
Sodium Chloride	1M
Ethanol (95%)	10%

pH was adjusted with HCl.

(e) Elution buffer (buffer E) pH 4.5

Sodium dihydrogen phosphate	100 mM
Tris HCl (pH 8.0)	10 mM

Urea	8 M
Triton X-100	0.1%
Sodium Chloride	1M

pH was adjusted with HCl.

4. **PBMC culture media**

RPMI 1640 medium

RPMI 1640 powder (Sigma)	One vial
HEPES (25mM)	5.95 gm
Sodium bicarbonate (20 mM)	2.0 gm
Penicillin-streptomycin (200X)	5 ml
Gentamicin (40mg/ml)	1.25 ml
ß- Mercaptoethanol	20 mM
Triple distilled water	upto 1000 ml

Stirred and filtered through 0.22 µ membrane filter, stored at 4°C.