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Expression Profile of Toll-Like Receptor mRNA in an Indigenous Aseel Breed of Chicken in India

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Abstract: Expression profile of chicken six Toll-like Receptor (TLR) mRNAs were analyzed in the heterophils, lungs, liver, spleen, duodenum, caecal tonsils and kidneys of 12-weeks old indigenous village chicken in India, using reverse transcription polymerase chain reaction (RT-PCR). TLR 2 type 1 mRNA was expressed in lungs, liver, spleen, duodenum and caecal tonsils. TLR2 type 2 mRNA was expressed only in the lungs. TLR 3 mRNA was expressed in lungs, liver, spleen and caecal tonsils. TLR 4 mRNA was expressed only in lungs, liver and spleen. TLR 5 and TLR 7 mRNAs were expressed in all the tissues examined. With respect to tissues, heterophils and lungs expressed all the TLR mRNAs examined while kidneys expressed only TLR 5 and TLR 7 mRNAs. All the TLR amplified PCR products were partially sequenced and showed high homologies with the available chicken (commercial) TLR gene sequences. There could be a possibility of correlation between TLR mRNA expressions with higher levels of innate immunity seen in indigenous village breeds of chickens.

Key words: Toll-like receptors, expression profiles, RT-PCR, indigenous chicken

INTRODUCTION

Recognition of potential pathogenic microorganisms by the innate immune system is a mediated by cellular receptors known as the Pattern Recognition Receptors (PRRs), which include the Toll-like Receptors (TLRs) (Akira, 2004 and Beutler, 2004). The innate immune system uses these receptors to recognize evolutionarily conserved molecular motifs, the Pathogen Associated Molecular Patterns (PAMPs) of infectious microbes, chemicals, etc. The TLRs represents an evolutionarily conserved signaling system that is a major determinant of the innate immune and inflammatory responses.

Members of the TLR family, first discovered in *Drosophila* (Kopp and Medzhitov, 1999) are type 1 transmembrane receptors with significant homology in the cytoplasmic domain to the IL-1 receptor type 1. In humans and mice, 11 TLRs have been identified with each member recognizing and responding to different microbial components. TLR2, in association with TLR1 and/or TLR6 induces signals after exposure to bacterial lipoproteins and peptido glycans, whereas Lipopolysaccharide (LPS) from Gram-negative bacteria signals through TLR4. Double-stranded RNA stimulates TLR3, bacterial flagellin induces signaling through TLR5 and TLR9 mediates responses to the CpG motif of the bacterial DNA. Upon activation with the appropriate ligand, TLRs induce a range of responses including cell proliferation and production of various cytokines, chemokines or effector molecules including nitric oxide and reactive oxygen intermediates (Thoma-Uszynski *et al*., 2000 and Smith *et al*., 2003).

The heterophil is the major polymorphonuclear cell in birds with a functional capacity akin to that of the mammalian neutrophil. It has been shown that heterophils constitutively express TLR1/6/10, TLR2 type 1, TLR2 type 2, TLR3, TLR4, TLR5 and TLR 7 mRNA (Kogut *et al*., 2005). The broad TLR expression profile in heterophils reflects their principal role as first line effector cells in avian host induced defense against bacterial, viral, fungal and parasitic infections. Expression patterns of chicken TLRs varied with some of the TLRs like TLR1/6/10, TLR3, TLR4 and TLR5 being expressed in most tissues while others exhibited more restricted expression patterns such as TLR2 type 1, TLR2 type 2 and TLR7. Similarly distinct patterns of TLR expression were seen innate and adaptive immune cell types isolated from peripheral blood or spleen and with cultured cells of somatic or immunological origin (Iqbal *et al*., 2005). The tissue and cell distribution is an important characteristic of TLR function since it influences the capacity to detect microorganisms during their entry and growth in different target tissues. Distribution of TLR repertoire in chicken heterophils (Kogut *et al*., 2005) and in various chicken tissues, *ex vivo* sorted cell populations and cultured cell types has been studied by Iqbal *et al*. (2005).

The Aseel poultry breed is among the most important and renowned indigenous breeds of India. The indigenous breeds of village chickens are considered to be more disease resistant than their commercial counterparts. Artificial breeding for production did not segregate the disease resistance parameters in

commercial chicken production and hence they are immunologically under privileged. The resistance of village chickens to many diseases indicates that they are immunologically superior. However, it is difficult to ascertain whether a single or a set of gene (s) define this property to village chickens.

In the present study, we examined the expression profile of TLR mRNA using reverse transcriptase polymerase chain reaction (RT-PCR) in chicken heterophils and selected tissues from one breed of indigenous village chicken in India.

MATERIALS AND METHODS

Experimental chickens: Indigenous 3-4 weeks old village chickens were obtained from local sources and were maintained under clean conditions in wire cages and given *ad libitum* access to water and feed. The birds did not receive any vaccination. At 12 weeks of age, these chickens were used for both for collection of heterophils from blood and selected tissues such as lung, live, spleen, duodenum, caecal tonsil and kidney.

Isolation of peripheral blood heterophils: Avian heterophils were isolated from the peripheral blood following the procedure described by Kogut *et al.* (2005). Briefly, disodium Ethylene Diamine Tetra Acetic Acid (EDTA) -anti-coagulated blood was mixed with 1% methyl cellulose (Sigma Chemical Co., USA) at a 1.5: 1 ratio and centrifuged at 25 x g for 30 min. The serum and buffy coat layers were suspended in Hank's Balanced Salt Solution (HBSS) devoid of calcium and magnesium salts. This was added over a discontinuous Ficoll-Hypaque gradient (specific gravity 1.077 over specific gravity 1.199) and centrifuged at 250 x g for 60 min. After centrifugation, the interface and 1.119 band containing the heterophils was collected and washed twice in RPMI 1640 medium (Invitrogen, USA) and resuspended in fresh RPMI 1640. Cell viability was determined by trypan blue exclusion. The purity of the heteophil suspension was assessed by microscopic examination of stained cytospin preparations. Heterophil preparation obtained by this method were always >95% pure and viable. The cell concentration was adjusted to 1 x 10^6 heterophils/ ml and used for RNA extractions.

RNA extraction and RT-PCR: The heterophil RNA was extracted using TriZol (Life Technologies, USA) and 1 microgram of RNA was reverse transcribed using the RevertAid cDNA synthesis kit (Fermentas, USA) with ologo dT primers according to the manufacturer's instructions. All the RNA preparations were standardized by RT-PCR for beta actin and were free from DNA contamination as judged by lack of signal from non-reverse transcribed RNA with all the primer sets (data not shown).

Selected tissues such as lung, spleen, kidneys, caecal tonsils, duodenum and liver were also collected following humane sacrifice of the birds and used in RNA extraction and RT-PCR as described below.

The primers used for the amplification of various TLRs have been described by Kogut *et al.* (2005) and shown in Table 1. PCR was performed using the various primers and the Red Dye mix (Bangalore Genei, Bangalore, India) in 25 µl reaction volumes. PCR conditions were as follows, 1 cycle of 95°C for 2 min followed by 30 cycles of 30s at 95°C, 58°C for 1 min and 72°C for 2 min followed by 1 cycle at 70°C for 10 min using a Gradient Master Cycler (Eppendorf, Germany). The amplified products were analyzed by 2% agarose (Sigma, Poole, UK) gel electrophoresis in 1 x TAE buffer at 50 mA for 1 h and products visualized by staining with ethidium bromide (BioRad, USA) using 100 bp ladder as a DNA size marker.

Sequencing of TLRs genes from indigenous chickens: The TLR PCR products obtained from indigenous chickens were purified using the QIAQuick PCR purification kit (Qiagen, Germany) and sequenced using the Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA) and an automatic sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems). These sequences were compared with the sequences of commercial chickens available in GenBank for their nucleotide homologies.

RESULTS

The heterophils showed expression of all the TLR mRNAs examined (Fig. 1). The expression profile of other TLR mRNAs in the tissues analyzed are shown in Figure 2 and the amplification summary is presented in Table 2. TLR 2 type 1 mRNA was expressed in lungs, liver, spleen, duodenum and caecal tonsils. TLR2 type 2 mRNA was expressed only in the lungs. TLR 3 mRNA was expressed in lungs, liver, spleen and caecal tonsils. TLR 4 mRNA was expressed only in lungs, liver and spleen. TLR 5 and TLR 7 mRNAs were expressed in all the tissues examined.

With respect to tissues, heterophils and lungs expressed all the TLR mRNAs examined while kidneys expressed only TLR5 and TLR7 mRNAs.

The sequences of the TLR PCR products amplified had 90-100% nucleotide homology with the GenBank sequences (Table 3) from commercial chickens. All Aseel chicken TLR sequences have been submitted to the GenBank under accession numbers FJ 605353-FJ 605358.

DISCUSSION

Indigenous breeds of chickens are considered disease resistant than their commercial counterparts. Whether this is due to their immunological superiority or due to the lack of exposure to infectious agents due to their less intensive nature of rearing is still questionable.

Table 1: Primers used in the study for amplification of TLR mRNA by RT-PCR

Primer	Sequence (5' to 3')	Accession number	Nucleotide position
TLR2 type 1 FP	TTAAAAGGGTGTGCCAGGAG	AB050005	138-157
TLR2 type 1 RP	GTCCAAACCCATGAAAGAGC		389-408
TLR2 type 2 FP	AGGCACTTGAGATGGAGCAC	AB046533	36-55
TLR2 type 2 RP	CCTGTTATGGGCCAGGTTTA		330-349
TLR 3 FP	CCACTCTGGAAGAAAATGAGC	BI066273	3-23
TLR 3 RP	TCATTCTCACCGCTTTTCAG		606-625
TLR 4 FP	AGTCTGAAATTGCTGAGCTCAAAT	NM001030693	205-228
TLR 4 RP	GCGACGTTAAGCCATGGAAG		375-394
TLR 5 FP	CCACATCTGACTTCTGCCTTT	AJ626848	1351-1371
TLR 5 RP	TGCCACATGTTTTCTCCTAGGT		1580-1601
TLR 7 FP	GCCTCAAGGAAGTCCCCAGA	AJ632302	578-597
TLR 7 RP	AAGAAACATTGCATGGATTACGG		969-991
Beta actin FP	TGCTGTGTTCCCATCTATCG	L08165	152-171
Beta actin RP	TTGGTGACAATACCGTGTTCA		281-301

Table 2: TLR mRNA expression in selected tissues of 12-weeks old indigenous chickens

TLRs	Heterophils	Lung	Liver	Spleen	Duo	CT	Kidney
TLR2 type 1	+	+	+	+	+	+	-
TLR2 type 2	+	+	-	-	-	-	-
TLR3	+	+	+	+	-	+	-
TLR4	+	+	+	+	-	-	-
TLR5	+	+	+	+	+	+	+
TLR7	+	+	+	+	+	+	+

+: indicates expression of specific TLR mRNA in the cell/tissue analyzed; -indicates absence or low expression of specific TLR in the tissue analyzed

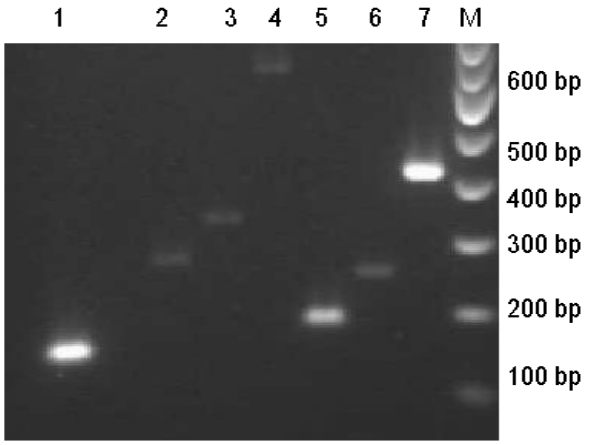

Fig. 1: TLR mRNA expression in the heterophils of 12-weeks old indigenous chickens. Lanes1-7 depicts expression of actin, TLR2 type 1, TLR 2 type 2, TLR3, TLR4, TLR5 and TLR 7 in that order. M shows 100 bp ladder, Note amplified products in all lanes 1, 2, 3, 4, 5, 6 and 7

Expression profiling for the presence of various TLRs mRNAs in different cells and tissues is an important feature that would characterize the ability of these cells and tissues in detecting pathogens. TLR expression profiles are also suggestive of an individual's ability to respond to challenge. Expression profiling of TLR mRNAs are essential before the application of TLRs agonists as vaccine adjuvants or for therapeutic targeting (Uematsu *et al*., 2004; Weeratna *et al*., 2005).

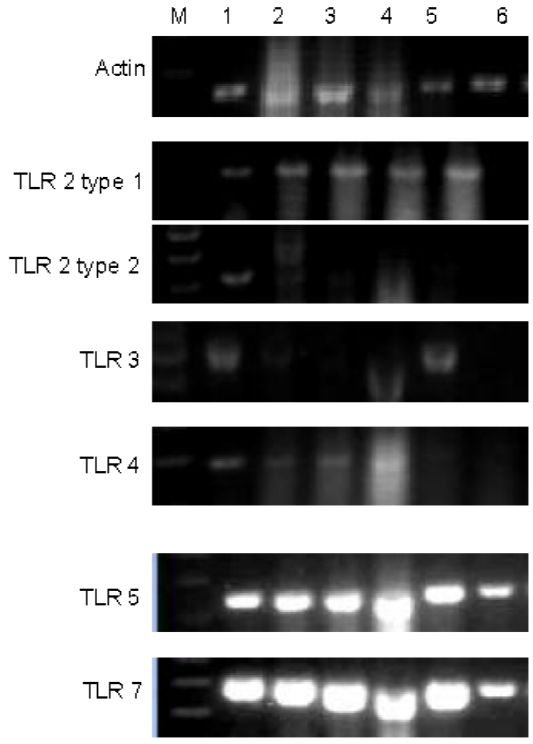

Fig. 2: Expression profiles of TLRs from selected tissues of 12 -weeks old indigenous chickens. Lane 1: Lung, Lane 2: Liver, Lane 3: Spleen, Lane 4: Duodenum, Lane 5: Caecal tonsils and Lane 6: Kidneys

Table 3: Percent nucleotide homologies of TLR mRNAs partial sequence from indigenous chickens (this study) as compared to commercial chicken sequence available in GenBank

TLR gene sequences from indigenous chickens	Homology to Accession number	Percent homology
TLR2 type 1	AB050005.2	98
TLR 2 type 2	FJ197821.1	96
TLR 3	NM001011691.2	90
TLR 4	FJ197822.1	95
TLR 5	EU052290.1	100
TLR 7	AJ627563.1	99

Expression of a wider repertoire of TLRs in tissues that interface between host and pathogen could be attributed to greater host resistance. If expression profiles of these TLR mRNAs are indicative of a heightened ability to respond to appropriate PAMPs, it seems likely that the lungs of indigenous chickens are very sensitive to all TLR ligands by virtue of their wide repertoire of TLR expression as this tissue interfaces with pathogens that enter through the respiratory systems.

The same could be true for heterophils which acts as a first line of defense to most infections. Kogut *et al.* (2005) demonstrated that heterophils constitutively expressed TLR1/6/10, TLR2 type 1, TLR2 type 2, TLR3, TLR4, TLR5 and TLR7 mRNA using day-old Leghorn chickens (Hy-Line W-36). Iqbal *et al.* (2005) also studied expression patterns of chicken TLRs in tissues, immune cell subsets and cell lines using inbred 8 weeks-old; line 7_2 White Leghorn (WLH) chickens. They found that heterophils expressed message for all of the known TLRs with TLR 1/6/10, TLR2, TLR4 and TLR5 being most prominent, moderate expression for TLR3 and low but detectable expression for TLR7. The broadest range of TLR expression occurred on cell subsets representing the innate immune system.

Iqbal *et al.* (2005) observed that WLH chickens showed expression of all the TLR mRNAs, examined in this work, in liver, spleen, duodenum, caecal tonsils and kidney while only TLR2 type 1 and type 2 showed a +/- expression in lungs. In the indigenous chickens, contrary to expectations, TLR mRNAs were not expressed in some tissues. However, TLR5 and TLR7 amplified products were more intense (in comparison to other PCR products) in the indigenous chickens while in the previous study with WLH chickens (Iqbal *et al.*, 2005), the relative level of expression of these TLR mRNAs was only + or ++ in a scale up to ++++. These findings highlight the fact that tissue-specific differences do exist in the TLR mRNA expression profiles between types of chickens. Another point for consideration is that Iqbal *et al.* (2005) used 8 weeks old chickens while our indigenous chickens were 12 weeks old. Further studies are needed to confirm these observations.

The kidneys expressed the lowest repertoire of TLR mRNAs further highlighting the susceptibility of this organ to most infectious agents. Expression of TLR5 and TLR7 mRNAs also suggests the ability of all tissues to respond to bacterial flagellin or single stranded RNA better than double stranded RNA (TLR 3) or lipo polysaccharide (TLR 4). All the amplified TLR products were confirmed using sequencing which showed high homologies with existing sequences available in GenBank.

The importance of an organ for immune defense is not dependent only on the expression on TLR mRNA's, since most tissues consist of many different cell types and a minor population of a specific cell type might be important in immune recognition. It should also be borne in mind that there could be differences associated with host genetics such as TLR4 polymorphism and environmental factors (Leveque *et al.*, 2003; Dil and Qureshi, 2002). Further work is needed to quantify the levels of mRNAs using quantitative real time PCR to elucidate the differences between the indigenous and commercial breeds of chicken.

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