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Constitutive expression of ch-TLR 4, IL-1β and IFN-γ in Aseel and Kadaknath chick peripheral blood mononuclear cells

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ABSTRACT

Immune system operates through innate and adaptive arm to fight against invading pathogens and confers protection to the host. Innate and adaptive arm differs in the two main cardinal attributes *i.e.*, specificity and memory. Innate arm is non-specific arm devoid of memory while adaptive arm operates with specificity and have memory. Earlier innate arm of the immune system was viewed as of limited role and mainly concerned with initial containment of the invading pathogen before adaptive arm comes into play. However, both the arms work in highly coherent manner to thwart pathogenic attack. In the recent past, the role of components of innate arm in generation of adaptive immune response has been established more precisely. By virtue of their role as "Antigen Presenting Cells", innate cells present antigenic peptides to Th and Tc cells. Moreover, a good number of pro-inflammatory cytokines are also secreted that has profound effect on subsequent adaptive response. This improved understanding of underlying mechanism allows assessing basal constitutive expression of TLRs and other important immune markers as a novel way of evaluating general immune competence of the host. Aseel and Kadaknath, Indian native breeds of chicks are considered to be more immune competent than the commercial breeds. This study was designed to assess basal constitutive expression of ch-TLR 4, IL-1b and IFN-γ in peripheral blood mononuclear cells (PBMCs) of Aseel and Kadaknath using real time PCR. PBMCs isolated from 21 days old Aseel chicks had 1.28, 1.59 and 2.38 times higher constitutive expression of ch-TLR4 and IL-1β, respectively than PBMCs isolated from Kadaknath. Thus, basal constitutive expression of these two innate markers was found to be relatively higher in PBMCs from Aseel than that of Kadaknath.

Key words : Chick, immune, poultry

In the last few decades, innate immune system has acquired centre stage of research interest. Innate arm initiates operation after recognizing highly conserved molecular patterns ("Pathogen associated molecular patterns" (PAMPs)) present in micro-organisms *viz.*, LPS, lipo-proteins, LTA, lipoarabino-mannans, flagellin, ds RNA, bacterial DNA, Zymosan etc., (Aderem and Ulevitch, 2000; Takeda *et al.*, 2003). These PAMPs are recognized by receptors called Pattern recognition receptors (PRRs). Among PRRs, Toll like receptors (TLRs) are of prime importance (Takeda *et al.*, 2003). Recognition of PAMPs by TLRs initiates a signaling cascade that involves a number of proteins including transcription factor NF-kB and AP-1 which induce the secretion of pro-inflammatory cytokines like IL-1, IL-6, IL-12 etc. and thereby effector cytokines that directs the adaptive immune response (Andrem and Ulevitch 2000). Applications of selective breeding in fowl have brought significant improvement in production traits of the fowl in comparison to their native counterparts. However, this has largely compromised with the immune competence of the fowl which is worrying in the wake of pathogenic

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threat faced by fowls. Thus, scientists are working hard to enhance the genetic resistance of fowl with optimum production performance. This needs exploration of genetic variability in available germplasm so that they could be incorporated in future selective breeding programmes. In fowl, most of the research work in this direction had been carried out in the fowl like leghorn or synthetic broiler lines and reports on these parameters from Indian native breeds like Aseel and Kadaknath, believed to exhibit more genetic resistant, is largely missing. High immune competence of these Indian native breeds had been reported on the basis of resistance to subgroup A Rous sarcoma virus (Rout *et al.*, 1992), high classical complement pathway activity and humoral immune response to SRBCs (Baelmans *et al.*, 2005) and higher serum nitric oxide levels (Ramasamy *et al.*, 2011). To have further insight on the immune competence of these Indian native breeds of fowl, this study has been designed to evaluate the constitutive expression of ch-TLR4, IL-1b and IFN-γ.

MATERIALS AND METHODS

Day old chicks of Aseel and Kadaknath breeds were purchased from the CPRS (Central Poultry Research Station), Anand. Chicks were maintained in the Poultry Demonstration Unit, Veterinary College, SDAU under standard management and during entire experimental period they were provided ad-libitum feeding & watering. Approx. 1.5 ml blood from Aseel and Kadaknath chicks were collected aseptically in heparin from 4 birds of both groups on 21th day of their age. Individual blood sample was mixed with PBS (1:1 dilution), 3 ml of this blood – PBS mixture was layered carefully on 3 ml of Histopaque (1.077, sigma-Aldrich) and centrifuged at 1800 rpm for exactly 20 minutes in swing out rotors. White ring at the interface containing mononuclear cells (PBMCs) was carefully aspirated and washed twice with HBSS at 800-1000 rpm for 10 minutes. PBMCs @ 1 X 10^6 were subjected to RNA isolation, c-DNA preparation and relative quantitation using Real time PCR (ABI, 7500). The primers used in the study were designed with the help of online available softwares (Table 1). The primers were of small amplicon size (100-300 bp), amplified at 58^0 C and showed primer efficiency in the range of 1.8-2.0. β-actin gene was taken as housekeeping gene for this study. The reaction conditions were standardized with SYBR green chemistry and all the primers produced a single peak in dissociation curve analysis confirming the specificity of the products. To determine the relative expression of genes under study between Aseel and Kadaknath breeds, the constitutive expression in PBMCs from Kadaknath chicks was considered as unit for respective genes under comparision.

Table 1 List of primers used in the study

Target genes	Primer	Amplicon size (bp)
Ch-TLR 4	F: CCA CCC TGG ACT TGG ACC TCA G = 22 R: AAG GCT GCT AGA CCC AGG TGC T = 22	202
Ch- IL-1 β	F: GCC TTC CGC TAC ACC CGC TC = 20 R: ATG TCC CGC ATG ACG TCG GC = 20	293
Ch- IFN-γ	F: ACC TGG CCA AGC TCC CGA TGA = 21 R: ACT CGG AGG ATC CAC CAG CTT CT = 23	95
β-actin	F: GCACCACACTTTCTACAATGAG= 22 R:ACGACCAGAGGCATACAGG= 20	184

RESULTS AND DISCUSSION

Constitutive expression of Ch-TLR4, IL-1β and IFN-γ

PBMCs isolated from both the Indian native breeds were found to have constitutive expression of ch-TLR 4, IL-1β and IFN-γ. PBMCs isolated from Aseel recorded higher expression for all the genes under study. In comparison to PBMCs from Kadaknath, PBMCs from Aseel were found to have 1.28, 1.59 and 2.38 times higher expression of ch-TLR4, IL-1β and IFN-γ, respectively (Table 2).

Table 2 Constitutive expression of ch-TLR4, IL-1β and IFN-γ in PBMCs of Aseel and Kadaknath chicks

Parameters	Target genes		
	TLR 4	IL-1β	IFN-γ
Aseel Average delta Ct* values (n=3)	-4.02	-3.84	-10.31
Kadaknath Average delta Ct* values (n=3)	-4.38	-4.51	-11.57
ddCt**	0.36	0.67	1.25
Level of relative expression in Aseel in comparison to Kadaknath	1.288	1.59	2.38

*delta Ct = Ct of housekeeping gene-Ct of Target gene
** dd Ct = delta Ct of target gene (Aseel) - delta Ct of target gene (Kadaknath)
Relative expression was calculated using IF command in excel= IF(V4>0,2^ddCt,-(2^-ddCt))

Innate arm of the immune system acts non-specifically and work in no time following their interaction with immune system. Cells of Innate immune arm plays major role in intitiation of immune response by functioning as "antigen presenting cells" which not only process and present antigenic peptides to T helper cells but also help them by producing several pro-inflammatory cytokines (Akira and Hemmi, 2003). Thus, the cell of this non-specific arm plays a major role in shaping subsequent adaptive immune response. These cells of innate arm recognize invading pathogen by the help of Pattern recognition receptors (one of them is Toll like receptors-TLRs) which detect structural motifs associated with microbes called "Pathogen Associated Molecular Patterns" (Medzhitov and Janeway, 2000). Examples of PAMPs are LPS, LTA, Flagellin, double or single stranded RNA, bacterial DNA or CpG etc. Interaction of innate cells with these microbial products could lead to up-regulation or down regulation in expression of various TLRs, pro-inflammatory cytokines and various co-stimulatory molecules (Andrem and Ulevitch 2000). Selective breeding in the past have enhanced the production trait in commercial poultry population but at the same time inversely affected their immune competence (Pinard *et al.*, 1998). The improved understanding of innate arm could also assist poultry scientists to meticulously look for desired genes to strengthen the genetic resistance in poultry. Keeping these facts in mind, this study was conducted on two Indian native breeds of fowl i.e., Aseel and Kadaknath which are reported to have better abilities to fight infectious diseases. An avian orthologue of mammalian TLR 4 was characterized by Leveque *et al.*, (2003). Interaction of ch-TLR 4 with its ligand leads to up-regulation of various immune related genes like IL-1β, IFN-γ, IL-10, CD80 and MHC II etc. (St Paul *et al.*, 2012) which directly influences the outcome of immune response. In the chicken, an IL-1-like biological activity was first demonstrated in supernatants of LPS-stimulated adherent spleen cells (Hayari *et al.*, 1982) and is considered as an important pro-inflammatory cytokine. Similarly, Interferon-γ (IFN-γ) represents the major macrophage-activating factor (MAF), exhibits antiviral activity and drives Th1 immune responses (Boehm *et al.*, 1997; Farrar and Schreiber, 1993). Ch IFN-γ is also known to prime heterophils for a significantly strengthened respiratory burst to subsequent stimulation with

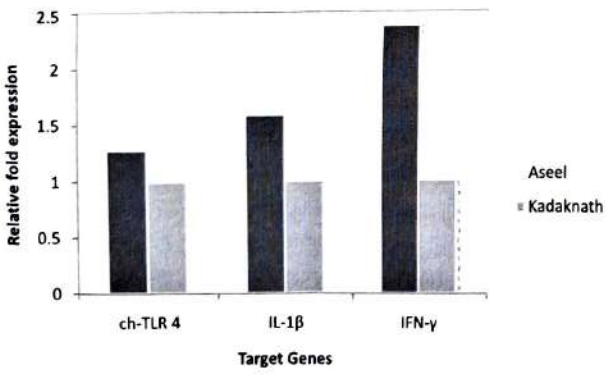

Plate 1 Relative expression of ch-TLR 4, IL-1β and IFN-γ genes in Aseel in comparison to Kadaknath chicks

opsonized zymosan (Kogut *et al.*, 2012). Th1 skewed response characterized by dominance of IFN-γ production has been correlated with resistance to MDV, Infectious Bronchitis, NDV and Salmonellosis in chicken (Zekarias *et al.*, 2002). Thus, constitutive expression of these three important genes *i.e.*, ch-TLR4, IL-1β and IFN-γ in PBMCs from these breeds were studied which is supposed to simply reflects general immune competence of the host. In the present study, the constitutive expression of all the three genes was detected in PBMCs isolated from both the Indian native breeds of fowl. In comparison to Kadaknath, PBMCs from Aseel were found to have 1.28, 1.59 and 2.38 times higher expression of ch-TLR4, IL-1β and IFN-γ, respectively. Contrary to our reports, Ramasamy *et al.*, (2010) reported significantly higher expression of TLR 4 and TLR 5 in heterophils from Kadaknath than in heterophils of Aseel chicks. The difference observed in the expression level in expression of ch-TLR4 in their report might be due to difference in target cells used in the studies i.e, PBMCs and heterophils. Thus, these reports indicated towards difference in expression of TLRs in different immune compartments. Differential responsiveness in the expression of various important immune related genes has been correlated with the disease susceptibility or resistance pattern of the host (Adams *et al.*, 2009). They reported that in response to low pathogenic AIV H11N9 infection, the expression of IL-1β, IFN-γ and IL-6 in chicken PBMC were found to be significantly up-regulated whereas in duck PBMC expression levels were minimal or unchanged. These results showed distinct PBMC expression patterns of pro-inflammatory cytokines and IFNs between species affecting clinical outcome. Moreover, Nerren *et al.*, (2009) reported the difference in TLR 15 gene expression by heterophils of lines A (Salmonella resistant) and B (Salmonella susceptible) which likely accounts for some of the observed differences between the lines in their susceptibility to infection. Constitutive expression of these immune markers observed in the present study give support to the report of Rout *et al.*, (1992) who injected 0.2 ml of Rous sarcoma virus containing 1000 pock forming unit /ml via chorio-allantoic membrane into the 11 day old embryos of Kadaknath, Aseel and White leghorn breeds of fowl that hatched subsequently. This study, intending to ascertain the genetic susceptibility of these breeds to Rous sarcoma virus concluded that Aseel and Kadaknath breed were 6 and 3 times less sensitive than white leghorn. Moreover, Kundu *et al.*, (1999) also recorded highest HA antibody titre in Aseel on 12th and 19th day post immunization with SRBCs than breeds like Kadakanath, Frizzle, Dahlem Red, White Leghorn, Synthetic dam line broiler (SDL) and Naked Neck.

CONCLUSION

Higher constitutive expression of ch-TLR4, IL-1β and IFN-γ observed in the PBMCs from Aseel chicks reflects towards better immune competence of Aseel than Kadaknath chicks. Since, immune response is governed by multiple genes, thus comprehensive trials with suitable infection model are warranted to test this notion further. The advantages of identifying and incorporating better alleles could be immense in the poultry industry, which is facing severe pathogenic challenges with devastating impact.

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