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ORIGINAL RESEARCH

# Evaluation of the constitutive expression levels of *ch-TLR 3, ch-TLR 4, ch-TLR 15* and *ch-TLR 21* genes in the Peripheral Blood Mononuclear Cells of native Indian poultry breeds, Aseel and Kadaknath

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## Abstract

**Aim:** To assess the basal constitutive expression levels of *ch-TLR3, ch-TLR 4, ch-TLR 15* and *ch-TLR 21* in the peripheral blood mononuclear cells (PBMCs) in Aseel and Kadaknath chicks (Indian native poultry breeds) and to evaluate the differences in their general innate immune competence.

**Materials and Methods:** PBMCs were isolated from 21 day old Aseel and Kadaknath chicks (n=4) and were subjected to RNA isolation and cDNA synthesis. The basal expression of *ch-TLR* 3, 4, 15 and 21 was studied using real time PCR with SYBR green chemistry using 18 S-rRNA as the housekeeping gene.

**Results:** PBMCs isolated from Kadaknath chicks exhibited a significantly higher ($p<0.05$) constitutive expression of *ch-TLR 3, ch-TLR 15* and *ch-TLR 21* genes when compared to Aseel chicks. In comparison to Aseel, Kadaknath chicks recorded 14.774, 7.182 and 3.507 fold higher expressions of *ch-TLR 3, ch-TLR 15* and *ch-TLR 21* genes, respectively. In contrast, the constitutive expression of *ch-TLR 4* was found to be higher (by 1.733 fold) in Aseel chicks.

**Conclusion:** Our results indicate that Kadaknath chicks are equipped with a better innate immune competence in comparison to Aseel chicks.

**Keywords:** Aseel, *ch-TLR3, ch-TLR4, ch-TLR15, ch-TLR21*, Kadaknath, Innate immune competence

## Introduction

Innate arm of the immune system, once considered to play only a limited role in eliciting a protective immune response, has now acquired a centre stage and is now believed to play a pivotal role in the initiation as well as shaping the subsequent adaptive immune responses [1]. Cells of the innate arm are capable of recognizing a large number of invading pathogens using only a few germline encoded pathogen recognition receptors (PRRs), out of which the Toll like receptors (TLRs) are of prime importance [2]. TLRs recognise molecular patterns known as "Pathogen associated molecular patterns (PAMPs)" that are unique to microbes *viz.,* Lipoteichoic acid (LTA), double stranded RNA, lipopolysaccharide (LPS), flagellin, unmethylated Cytosine triphosphate-phosphodiester link-Guanine triphosphate (CpG) etc. which are recognised in chicken by *ch-TLR2, ch-TLR 3, ch-TLR 4, ch-TLR 5* and *ch-TLR 21* genes [3]. This pathogen sensing mechanism enables innate arm to recognise majority of the pathogens only with the help of a small repertoire of receptors. The efficiency of this system can be appreciated by the fact that recognition of LPS through TLR4 allows innate arm to recognise almost all of the gram negative bacteria. Interaction of these TLR ligands with the respective TLRs leads to either upregulation or downregulation of various immune related genes which have an important bearing on shaping subsequent adaptive responses into a more precise response [4].

Extensive breeding and selection programmes to improve economic attributes in commercial poultry flocks have compromised its innate immune competence in comparison to their native counterparts. The discoveries related to functioning of innate arm of the immune system have widely attracted the attention and infused a lot of interest in poultry scientists whose target is to improve the innate resistance of commercial flocks by looking for a better germplasm [5]. Generally, the immune competence of birds is assessed based on their level of complement activity or antibody response to Sheep RBCs (SRBCs). In the recent past, after the establishment of pivotal role of TLRs in pathogen recognition and generation of both innate and adaptive immune response, the constitutive and inducible expression of TLRs and other related genes has been correlated with general immune competence of the

**Table-1.** List of primers used in the study

| Target Gene | Primer Sequence | Amplicon Size | Accession No |
|---|---|---|---|
| *ch-TLR 3* | F: 5'-GTA AAG TGC CCC CTG TGC CGA A-3'<br>R: 5'-AGC TCG GTA CTC CCT GCG CT-3' | 135 | JF273967.1 |
| *ch-TLR 4* | F: 5'-CCA CCC TGG ACT TGG ACC TCA G-3'<br>R: 5'-AAG GCT GCT AGA CCC AGG TGC T-3' | 202 | NM_001030693.1 |
| *ch- TLR 15* | F: 5'-ACC AGA CAG ACG GGG CTC GC-3'<br>R: 5'-CAA AGC GCC AGC ATA AGC CCG-3' | 137 | NM_001037835.1 |
| *ch-TLR 21* | F: 5'-TGA GGA TGA TGG AGA CAG CGG AGA-3'<br>R: 5'-AGC GCG ATG GCA TGT GGT GG-3' | 238 | NM_001030558.1 |
| *18 S- rRNA* | F: 5'-GAT GCC GAC TCG CGA TCC GG-3'<br>R: 5'-GCC GGG TGA GGT TTC CCG TG-3' | 186 | AF173612.1 |

**Table-2.** Constitutive expression of *ch-TLR 3, ch-TLR 4, ch-TLR 15* and *ch-TLR 21* in PBMCs from Aseel and Kadaknath chicks

| Parameters | Target genes | | | |
|---|---|---|---|---|
| | ***ch-TLR 3*** | ***ch-TLR 4*** | ***ch-TLR 15*** | ***ch-TLR 21*** |
| Aseel chicks, Average delta Ct* values (n=4) | -14.97± 0.44 | -10.169± 0.30 | -9.944± 0.51 | -13.018± 0.46 |
| Kadaknath chicks, Average delta Ct* values (n=4) | -11.08± 0.853 | -10.963± 0.59 | -7.1± 0.45 | -11.208± 0.20 |
| ddCt** in Kadaknath chicks in comparison to Aseel chicks | 3.885 | -0.794 | 2.844 | 1.81 |
| Level of relative expression in Kadaknath chicks in comparison to Aseel chicks | 14.774 | -1.733 | 7.182 | 3.507 |

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

fowl [6]. A better disease tolerance of Aseel and Kadaknath, Indian chicken breeds, in comparison to their commercial counterparts is well documented [7,8]. Nevertheless, majority of the studies that were aimed at determining the immune competence, on the basis of constitutive and inducible expression of ch-TLRs and other related genes, in fowls were conducted in commercial breeds and the knowledge related to the expression of these innate immune markers in native poultry breeds such as Aseel and Kadaknath is largely unknown.

Thus, we designed this study with an objective to evaluate the constitutive expression of genes that encode four important chicken TLRs i.e., *ch-TLR 3, ch-TLR 4, ch-TLR 15* and *ch-TLR 21* in the peripheral blood mononuclear cells (PBMCs) of Indian native breeds; Aseel and Kadaknath.

## Materials and Methods

**Ethical approval:** This study was conducted as per the provisions of the Institute Animal Ethics Committee.

**Chicks:** Day old chicks belonging to Aseel and Kadaknath breeds were purchased from the Central Poultry Research Station, Anand. Chicks were maintained in the Poultry Demonstration Unit, Veterinary College, SDAU-Sardarkrushinagar under standard managemental procedures and during the entire experimental period they were provided with *ad-libitum* feed and water.

**Sample collection:** Approximately 1.5 ml of blood from four chicks of either sex from Aseel and Kadaknath breeds was collected aseptically from the jugular vein into heparin coated vials on the 21$^{th}$ day of their age.

**Peripheral blood mononuclear cells (PBMCs) isolation:** Individual blood samples were mixed with PBS (1:1 dilution), 3 ml of this blood – PBS mixture was then layered carefully onto 3 ml of Histopaque (1.077, sigma-Aldrich) and centrifuged at 1800 rpm for 20 minutes in swing out rotors. White ring at the interface containing the mononuclear cells/PBMCs was carefully aspirated and washed twice with HBSS followed by centrifugation at 800-1000 rpm for 10 minutes.

**Relative expression study:** PBMCs @ 2 X 10$^6$ were subjected to RNA isolation, c-DNA preparation and relative quantification using real time PCR (ABI, 7500). The primers used in the study were designed using the softwares available online (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. *18S rRNA* was considered as the housekeeping gene for this study. The reaction conditions were standardized with SYBR green chemistry and all the primers produced a single peak in the dissociation curve analysis confirming the high specificity of the products. To determine the relative expression levels of genes investigated between Aseel and Kadaknath breeds, the constitutive expression in PBMCs from Aseel breed was considered as a unit (control) for respective genes under comparison.

**Statistical analysis:** Data were analysed using WASP 1 software available online in the statistical package at ICAR-Goa complex site (http://www.icargoa.res.in/wasp/rbd1.php).

## Results

PBMCs isolated from both the Indian native breeds were found to have constitutive expression of *ch-TLR 3, ch-TLR 4, ch-TLR 15* and *ch-TLR 21.* In comparison to Aseel chicks, PBMCs from Kadaknath chicks showed significantly higher ($p<0.05$) constitutive expression of *ch-TLR 3, ch-TLR 15* and *ch-TLR 21.* Considering unit expression of *ch-TLR* genes

investigated here in Aseel chicks, Kadaknath chicks recorded 14.774, 7.182 and 3.507 fold higher expressions of *ch-TLR 3, ch-TLR 15* and *ch-TLR 21* genes, respectively. However, an exception was that the constitutive expression level of *ch-TLR 4* was found to be apparently higher in PBMCs isolated from Aseel chicks than that of Kadaknath chicks. Though non-significant, the expression of TLR 4 was found to be 1.733 times higher in Aseel than Kadaknath chicks PBMCs (Table-2).

## Discussion

The discovery of toll-like receptors (TLRs), particularly on the cells of innate arm of immune system [9,10] and their critical role in the providing immunity has aroused intense curiosity in the scientific arena and the studies conducted in the recent years on this subject has greatly improved our understanding of the functioning of immune system [11]. A better understanding of innate arm could also assist poultry scientists to meticulously look for desired immune-related genes with a potential to be incorporated in marker assisted selective breeding programmes. 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 enhanced abilities to fight infectious diseases when compared to abilities of the commercial breeds [12, 13].

TLRs are a group of highly conserved and germline encoded pattern recognition receptors. Chicken genome has orthologs of mammalian *TLR 1, TLR 2, TLR 3, TLR 4, TLR 5* and *TLR 7* whereas orthologs of *TLR 8, TLR9* and *TLR 10* appears to be either defective or missing [14]. TLR 3 recognises double stranded RNA intermediate formed during viral replication phase and directs the immune system to mount an anti-viral response [15]. TLR 4 recognises LPS from gram negative bacteria [16]. An avian orthologue of mammalian TLR 4 was characterized [17]. TLR 15 was discovered in chicken and considered to be an avian specific member of the TLR family. The role of TLR 15 in innate defence against *Salmonella* Enterica serovar Typhimurium is suggested [18]. Mammals recognise and respond to CpG ODN through TLR 9 which was found missing in the chicken genome. However, chicken showed somewhat similar responsiveness to CpG ODN. Later on, the recognition of CpG ODN was reported to be mediated by TLR 21 [19]. The role of TLR 3, TLR 4, TLR 15 and TLR 21 in host defense against viral and bacterial pathogens is well documented [20-23]. These earlier findings formed the premise for selecting *ch-TLR 3, ch-TLR 4, ch-TLR 15* and *ch-TLR 21* genes and to study their constitutive expression in PBMCs of Indian native Aseel and Kadaknath breeds which enables us to better under-stand the general immune competence of the native Indian poultry breeds. The PBMCs were collected from 21 day old chicks which coincide with the maturation of bursa at three weeks of their age and thereby lead to an acquisition of better immune competence.

In the present study, PBMCs isolated from 21 day old Kadaknath chicks had significantly higher *ch-TLR 3, ch-TLR 15* and *ch-TLR 21* m-RNA transcript expression, whereas *ch-TLR 4* had apparently higher expression in Aseel. Differential expression of various important immune-related genes has been correlated with the disease susceptibility or resistance pattern of the host [24]. The difference in *ch-TLR 15* gene expression by heterophils of lines A (Salmonella resistant) and B (Salmonella susceptible) is speculated to account for some of the observed differences between the lines in their susceptibility to infection [25]. The bacterial load in susceptible chickens was significantly higher than that in the resistant chickens and *ch-TLR4, ch-TLR2-1* and *ch-TLR21* expression was highly diminished in the leukocytes of susceptible chickens when compared with those of resistant chickens [26]. Higher expression of *ch-TLR 4, ch-TLR 15, ch-TLR 21* and a few other genes involved with TLR activation pathways was reported with *Salmonella* Enteritidis (SE) infection in chicken line A (resistant to *Salmonella* Enteritidis infection) [27]. The significantly higher expression of *ch-TLR3, ch-TLR 15* and *ch-TLR 21* in Kadaknath chicks, observed in the present study, is suggestive of a better immune competence of Kadaknath in comparison to Aseel chicks. In present study, constitutive expression of *ch-TLR 4* in PBMCs from Aseel chicks was found to be apparently higher than that of Kadaknath chicks. Contrary to our reports, significantly higher expression of *ch-TLR 4* and *ch-TLR 5* in heterophils from Kadaknath than in heterophils of Aseel, Necked neck, Dwarf and White Leghorn lines was reported earlier [28]. Contrasting observations in the expression level of *ch-TLR4* in the previous and our current reports might be due to difference in the target cells used in the two studies i.e., heterophils in the earlier study and PBMCs in our study. Nevertheless, these reports indicate a differential expression of TLRs in different immune compartments.

Poultry health is constantly under the threat of a wide array of pathogens of which viral and bacterial diseases have most devastating impact. The better immune competence in indigenous chicken is ascribed to higher complement activity, higher serum lysozyme level and antibody response [13,29]. The basal *TLR* mRNA expression profiles in different cells and tissues are suggestive of an individual's ability in responding to a challenge [30]. The present study indicates a better immune competence of Kadaknath breed to deal with a variety of the invading pathogens. In this context, it is important to note that we have not used any infectious model to ascertain the immune competence of these two breeds by exposing them to pathogenic challenge. Thus, ingenious experimental designs combined with the efficiency of latest tools and techniques are required to test this notion further.

## Conclusion

Higher expression of *TLR* genes has been

correlated with enhanced ability of the host to encounter invading pathogens. Thus, findings from the present study reflect a better immune competence of Kadaknath over Aseel chicks. However, considering the operational complexity of the immune response, further investigations are needed to fully ascertain how these differences in Aseel and Kadaknath chicks translate into development of an efficacious adaptive immune response. Future studies on Aseel and Kadaknath chicks along with other native breeds with target tissues like spleen, bursa, proximal GI tract, caecum etc. is highly warranted which will be of immense help in identifying important alleles involved in deciding resistance or susceptibility patterns of the host. We strongly believe that these future studies will pave way for the incorporation of better genetic resources in designing selective breeding programmes for efficient control and prevention of diseases.

### Authors' contributions

All authors contributed equally. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interest.

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