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Differential Expression of Cytokine in Kadaknath and Commercial Chicken Against Coccidial Challenge

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ABSTRACT

In the present investigation the relative expression of interleukins (IL)-1β, IL-2, IL-6, IL-17 and interferon (IFN)-Y genes were explored in response to coccidial challenge in Kadaknath (n=12), Cari-Vishal (n=12) and Cobb (n=12) broiler chicken using quantitative PCR. The intraepithelial lymphocytes cell of control, T_1 and T_2 on 4, 7 and 14d post infection (pi) were used as templates for expression of five inflammatory cytokines. The relative mean fold expression for ILβ1 ranged from 9.91± 0.14 (Kadaknath) to 119.01±0.29 (Cobb). The highest and lowest relative mean fold expression for IL-2 was observed to be 242.19±0.40 and 6.25±0.03 in Cobb at d4 and d14 pi, respectively. For IL-6 gene, the lowest relative mean fold expression was observed in Kadaknath (8.11±0.05) while the highest value was recorded in Cobb (99.04±0.92). The relative mean fold expression for IL-17 was 11.04±0.29 in Kadaknath and 197.40±0.37 in Cobb. For IFN-Y gene, the lowest relative mean fold expression was observed in Kadaknath (8.11±0.19), while the highest value was recorded in Cobb (84.74±0.25). In Cobb and Cari-Vishal broilers most cytokines (IL-1β, IL-6, IL-17 and IFN-Y) were observed to be transcribed to higher levels than Kadaknath chicken. It is concluded that the differential expression of cytokine genes in three genetic groups showed different degree of mucosal immune response to *Eimeria* infection and it depend upon the genetic background/ genotype of birds, coccidial dosage and age of infection.

Key words: Chicken, Coccidial challenge, Cytokines, Gene expression, Kadaknath.

INTRODUCTION

Genetic resistance to disease in chicken or any other species governed by the many genes and their interactions. This is indicated by the communication of the immune cells or membrane proteins such as molecules of Major Histocompatibility Complex (MHC), T-cells receptors (TCR) and immunoglobulins (B cell receptors and secreted proteins such as cytokines and antibodies). Genetic selections for high immune responsiveness and disease resistance can make improvement in the fitness traits of birds and also responsible for effectiveness of vaccination against diseases (Zacharias *et al*., 2002). Cytokines such as interleukins and interferon gamma (IFN-Y) are produced by T-helper-1 cells and are responsible for delayed hypersensitivity, macrophage activation and enhanced non-specific immunity to parasites (Hong *et al*., 2006 and Kim *et al*., 2012).

Cytokines are small glycoproteins (<30 kDa) molecules that mediate and orchestrate the complex events in the immune reaction, from the initiation of the acute phase reaction to the clonal expansion of effectors T and B cells. The availability of new technologies such as real-time quantitative PCR allows the estimation of expression of mRNA from cytokine genes without the need for protein or antibody. This has opened up a large area of possibilities to determine cytokine levels in disease, giving increased understanding of the mechanisms of both pathogenesis and immunity (Wigley and Kaiser, 2003).

MATERIALS AND METHODS

Experimental birds

The present investigation was conducted on Kadaknath (n=12), Cari Vishal (n=12) and Cobb (n=12) broiler chicken under the research project funded by Madhya Pradesh Biotechnology Council, Bhopal, Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, NanajiDeshmukh Veterinary Science University (NDVSU), Jabalpur.

Coccidial challenge

Chicks from each Kadaknath, Cari Vishal and Cobb broiler chicken were randomly divided into a control (C) group and two treatment groups (T_1 and T_2) comprising 12 chicks under each group. On arrival (day 1) chicks were wing-banded, weighed and randomly allocated to the treatments. During the entire experimental periods the standard chick diet free of any anti-coccidial additives and sanitized tap water were given to the birds *ad lib* throughout the experiment. Group

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T_1 and T_2 were challenged by gavaging 10,000 and 20,000 sporulated oocyst, respectively to each bird on 21st days of age. The control group was given 1 ml of Hanks Balanced Salt Solution (HBSS). On day 4, 7 and 14 post challenge, two birds from each group were sacrificed and lesion score, oocyst index, oocyst production (OPG) and mortality (if any) were recorded on these intervals.

Total RNA isolation, cDNA preparation and its quality check

Total RNA was extracted from intraepithelial lymphocytes using TRIzol method as per manufacturer's instructions (Rio *et al*., 2010). The concentrations of total RNA as quantified by spectrophotometer (ND 2000) ranged from 130 to 165 ng/µl. The OD 260/280 of these samples was just above 1.9. The DNase treated total RNA was converted into cDNA by using cDNA synthesis kit. Integrity and quality of cDNA were checked by GAPDH primer. Amplicon of size 264 bp on 2% agarose gel clearly showed no contamination of DNA, having good integrity of total RNA.

Primer selection and its specificity confirmation

Specific primers of cytokine genes IL-1β, IL-2, IL-6, IL-17 and IFN-Y were used for expression analysis of these cytokine genes (Table 1; Hong *et al*., 2006). The cDNA from 1000ng of total RNA extracted from intraepithelial lymphocytes cell of control, treatment group one (T_1) and treatment group two (T_2) on day 4,7 and 14 post challenge were used as templates for expression study IL-1β, IL-2, IL-6, IL-17 and IFN-Y genes. Amplification of all the gene fragments was confirmed by agarose gel electrophoresis (2%) of PCR product for all cDNA samples. Single band of the expected size, i.e., 244 bp for IL-1β, 256 bp for IL-2, 272 bp for IL-6, 292 bp for IL-17 and 259 for IFN-Y genes were observed on 2% agarose gel electrophoresis. The specificity of primers was also confirmed by real-time PCR dissociation curves. A primer concentration of 1.25 pmol was optimized for the amplification of all the gene fragments.

Real-time RT-PCR protocol

The real time RT-PCR was performed by SYBR green chemistry (ABI, USA) using cDNA as template for different genes. All PCR reactions were performed in triplicates with GAPDH as an internal control to normalize the amount of sample RNA in optical 96 well plate in final reaction volume of 20 µl. Thermal cycling condition comprised of initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing and extension at 60°C for 1 min. At the end of extension step, fluorescence was captured. At the end of each run, a melt-curve analysis (95°C for 15 s, 60°C for 1 min and increase of 0.5°C/5 s until 95°C) was performed to assess the specificity of the amplification. The specificity of the amplification products was also confirmed by the appearance of predicted-size fragments after 2% agarose gel electrophoresis.

After quantiûcation, the data were analyzed with the Sequence Detection System (SDS) software v1.3.0 (Applied Biosystems). Comparative C_T method was used for relative quantiûcation of the target cDNA relative to GAPDH cDNA (endogenous control) as per the standard methods (Livak and Schmittgen, 2001). The specificity of real time PCR amplification was determined on the basis of specific band size of the respective gene on 2% agarose gel electrophoresis.

Relative expression of cytokine genes

The efficiency of real-time PCR and relative quantification of target genes was calculated based on the methods described by Livak and Schmittgen (2001). The relative expression of cytokine genes in treatment groups (T_1 and T_2) was compared to that of control of respective breeds / lines and fold changes in gene expression were calculated using the Sequence Detection System (SDS) software v1.3.0 (Applied Biosystems). Fold changes were subjected to analysis of variance (ANOVA) using the MSTAT-C software to determine the effect of breed, coccidial dose and interval (day) on expression of cytokine genes.

RESULTS AND DISCUSSION

The relative expression of IL-1β, IL-2, IL-6, IL-17 and IFN-Y in Kadaknath, Cari-Vishal and Cobb broiler chicken in control and treatment group on day 4, 7 and 14 post challenge have

Table 1: List of primers specific to standard haplotypes of chicken MHC B-Lβ II family gene.

RNATarget		Primer sequences	Size (bp)
IL-1 β	Forward	5′-TGGGCATCAAGGGCTACA-3′	244
	Reverse	5′-TCGGGTTGGTTGGTGATG-3′	
IL-2	Forward	5′-TCTGGGACCACTGTATGCTCT-3′	256
	Reverse	5′-ACACCAGTGGGAAACAGTATCA-3′	
IL-6	Forward	5′-CAAGGTGACGGAGGAGGAC-3′	254
	Reverse	5′-TGCCGAGGAGGGATTTCT-3′	
IL-17	Forward	5′-CTCCGATCCCTTATTCTCCTC-3′	292
	Reverse	5′-AAGCGGTTGTGGTCCTCAT-3′	
IFN-Y	Forward	5′-AGCTGACGGTGGACCTATTATT-3′	259
	Reverse	5′-GGCTTTGCGCTGGATTC-3′	
GAPDH	Forward	5′-GGTGGTGCTAAGCGTGTTAT-3′	264
	Reverse	5′-ACCTCTGTCATCTCTCCACA-3′	

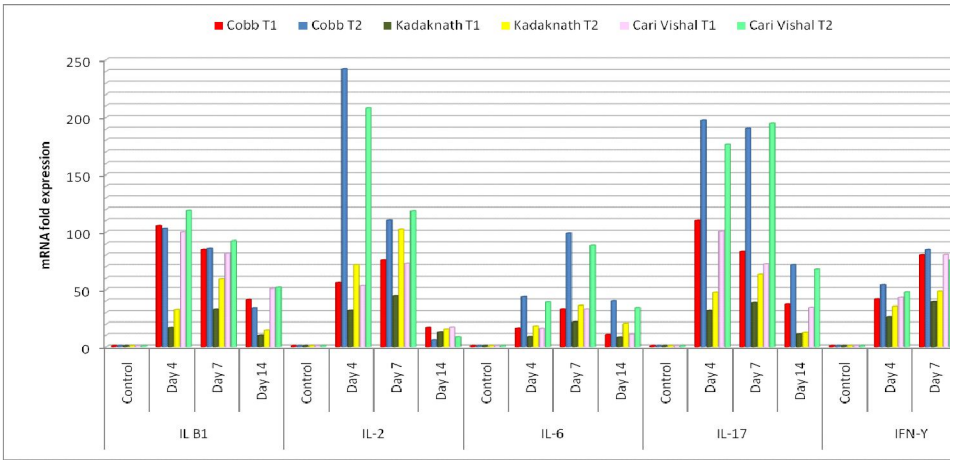

Fig 1: Relative expression of different cytokine genes in chicken on day 4, 7 and 14 post challenges.

Table 2: Least squares analysis of variance for Cytokine genes in Cobb, Kadaknath and Cari-Vishal chickens.

Cytokine genes		Source of Variation			
		Breed	Dose	Interval	Error
IL-1 β	MS	16147.34(2)	648.27(3)	2368.64(12)	0.44(36)
	F- Value	24.91**	0.27	5426.49**	
IL-2	MS	7754.89(2)	9663.97(3)	13131.02(12)	725.00(36)
	F- Value	0.80	0.74	18.11**	
IL-6	MS	2153.46(2)	4549.60(3)	1157.93(12)	22.47(36)
	F- Value	0.47	3.93*	51.54**	
IL-17	MS	35952.14(2)	14407.50(3)	6982.54(12)	160.00(36)
	F- Value	2.50	2.06	43.64**	
IFN-Y	MS	3561.87(2)	212.07(3)	1429.51(12)	4.01(36)
	F- Value	16.80**	0.15	356.69**	

*Significant (p<0.05),** Significant (p<0.01), Values in parentheses are degree of freedom.

been depicted in Fig 1. The least squares analysis of variance (Table 2) revealed highly significant (p<0.01) effect of breed for IL-1β gene and IFN-Y gene. The effect of dose was significant (p<0.05) for IL-6 gene and non significant for remaining cytokine genes. The effect of interval (post challenge) was found to be highly significant (p<0.01) for all five cytokine genes.

The LSM for relative fold expression of these five cytokine genes (IL-1β, IL-2, IL-6, IL-17 and IFN-Y) have been presented in Table 3 and depicted in Fig 1. The relative mean fold expression of IL-1β gene for T_1 (dose 1) and T_2 (dose 2) ranged from 9.91±0.14 (Kadaknath) to 105.78±0.52 (Cobb) and 14.47±0.28 (Kadaknath) to 119.01±0.29 (Cari-Vishal), respectively. The highest and lowest relative mean fold expression for IL-2 gene for T_2 (dose 2) was found to be 242.19±0.40 and 6.25±0.03 in Cobb at day 4 and 14 post challenge, respectively. However, the relative mean fold expression of IL-2 gene for T_1 (dose 1) was found to be maximum in Cobb (75.84±0.58) and minimum in Kadaknath (12.77±0.38) at day 7 and day 14 post challenge, respectively.

For IL-6 gene, the lowest relative mean fold expression was observed in Kadaknath (8.11±0.05) for dose 1 at day 14 while the highest value was recorded in Cobb (99.04±0.92) at day 7 for dose 2. The relative mean fold expression for IL-17 gene was found to be lowest with a value of 11.04±0.29 (dose 1, day 14) in Kadaknath and maximum was seen in Cobb i.e. 197.40±0.37 (dose 2, day 4). For IFN-Y gene, the lowest relative mean fold expression was found to be 8.11±0.19 (dose 1, day 14) in Kadaknath while the highest value was recorded in Cobb (84.74±0.25) for dose 2 at day 7 post challenge. These differences in expression of cytokine genes among the three genetic groups could be attributed to the fact that expression of cytokine genes varies depending upon the genetic background of birds, level of coccidial dosage, genotype of birds and age of infection (Schat and Davis, 1999; Zhang *et al*., 2012). The higher relative expression of all five cytokine genes in all the three genetic groups, confirming that these cytokine genes play an important role in the immune response to coccidiosis.

The upregulation of IL-10 gene (Table 3; Fig 1), as observed in the present study, was also reported by Rothwell *et al.* (2004). They measured the expression of mRNA for chIL-10 and other signature cytokines in gut and

Table 3: Average fold expression of cytokine genes in Cobb, Kadaknath and Cari-Vishal chicken.

Cytokine genes	Breed→	Cobb		Kadaknath		Cari-Vishal	
	Dose / Interval	Dose 1	Dose 2	Dose 1	Dose 2	Dose 1	Dose 2
IL-1 β	Day 4	105.78^{cA}±0.52	103.25^{dA}±0.20	16.79^{aB}±0.18	32.55^{fB}±0.34	100.07^{eA}±0.28	119.01^{bA}±0.29
	Day 7	84.74^{bB}±0.51	85.82^{bB}±0.54	32.82^{eA}±0.23	59.30^{cA}±0.28	81.28^{dB}±0.16	92.41^{aB}±0.46
	Day 14	41.06^{bC}±0.35	34.05^{cC}±0.11	9.91^{eC}±0.14	14.47^{dC}±0.28	50.91^{aC}±0.24	51.80^{aC}±0.52
IL-2	Day 4	55.90^{bAB}±0.53	242.19^{aA}±0.40	31.88^{bA}±1.09	71.50^{bA}±0.49	53.26^{bA}±0.30	207.93^{aA}±0.58
	Day 7	75.84^{abA}±0.58	110.27^{aB}±0.43	44.47^{bA}±0.43	102.53^{aA}±0.42	72.75^{abA}±0.16	118.60^{aB}±0.36
	Day 14	16.91^{ab}±0.29	6.25^{aC}±0.03	12.77^{aA}±0.38	15.40^{aB}±0.42	17.20^{aA}±0.06	8.48^{aC}±0.15
IL-6	Day 4	16.23^{bcB}±0.18	43.86^{ab}±0.18	8.60^{cB}±0.11	18.18^{bB}±0.10	16.16b^{cB}±0.19	38.98^{aB}±0.68
	Day 7	33.01^{cA}±0.12	99.04^{aA}±0.92	21.78^{dA}±0.33	36.00^{cA}±0.22	33.01^{cA}±0.24	88.64^{bA}±0.67
	Day 14	10.43^{eB}±0.44	39.94^{cB}±0.13	8.11^{eB}±0.05	20.47^{dB}±0.42	10.71^{bB}±0.32	34.17aB±0.48
IL-17	Day 4	110.00^{bA}±0.29	197.40^{aA}±0.37	31.77^{cA}±0.49	47.66^{cA}±0.41	100.77^{bA}±0.85	176.68aA±0.35
	Day 7	82.99^{bB}±0.29	190.67^{aA}±0.30	38.45^{cA}±0.30	63.55^{bA}±1.09	72.25^{bB}±0.83	194.69^{aA}±0.46
	Day 14	37.01^{bC}±0.22	71.50^{aB}±0.56	11.04^{cB}±0.29	12.46^{cB}±0.21	34.41^{bC}±0.50	67.64^{aB}±0.57
IFN-Y	Day 4	41.64^{cB}±0.35	54.00^{aB}±0.30	25.99^{eB}±0.56	35.38^{dB}±0.35	43.11^{cB}±0.98	48.00^{bB}±0.24
	Day 7	79.89^{bA}±0.34	84.74^{aA}±0.25	39.26^{eA}±0.45	48.67^{dA}±0.33	80.17^{bA}±0.52	75.84^{cA}±0.37
	Day 14	27.18^{cC}±0.20	31.55^{bC}±0.30	8.11^{eC}±0.19	17.26^{dC}±0.18	34.17^{bC}±0.21	41.35^{aC}±0.61

Values between columns (Lower case) and within column (uppercase) with different superscripts differed significantly ($p< 0.05$).

spleen of resistant (line CB12) and susceptible (line 15I) chickens during the course of an *E. maxima* infection. Further their findings were in accordance with the present results that susceptible chickens showed higher levels of this cytokine gene expression after infection in the small intestine than did resistant chickens. The upregulation of IFN-Y, IL-10 and IL-12 genes have also been reported by Hong *et al.* (2006) in intestinal lymphocytes following *E. acervulina*and *E. tenella*primary and secondary infection which is in agreement with present findings. They found that transcripts encoding the cytokines genes IFN-Y, IL-10 and IL-12 were increased following *E. tenella*primary infection. They also observed that cytokines IL-3 gene increased upto 327 fold following primary or secondary infection with both parasites. Similar findings were also reported by Gadde *et al.* (2011) in turkey poults. They found an increased expression of IFN-Y and IL10 genes on day 7 post challenge and IL1β and IL13 genes on day 7 post challenge following an oral infection with 12.5×10^3 oocysts of *Eimeria adenoeides.* Significant up-regulation of IL-10 mRNA in the caecum was also reported by Haritova and Stanilova (2012) in poultry with experimental coccidiosis. Zhang *et al.* (2012) also observed significant up regulation (156.1-1117.1-fold change) of IFN-α, IFN-β, IFN-Y, IL-1β and IL-12 genes in day-old chickens at 3 hour post infection in comparison to the different peak level times and relatively low expression of cytokine genes in the 3 week old chickens. Similar findings were reported by Al-Idreesi *et al*. (2013) who noticed significantly higher level of IFN-Y in the serum samples when compared with those of the control negative group. They observed that the serum levels of IFN-Y increased gradually after first and second dose of vaccine but increased drastically after challenge, as compared with control negative group. In contrast to the present findings, Kim *et al.* (2014) reported the down regulation of the chIL-17RA gene transcript in caecum tissues from chickens infected with *E. tenella*. These finding commensurate that the higher level of cytokine genes as observed in Cobb and Cari-vishal can be correlated to genetic susceptibility to coccidial infection.

CONCLUSION

The differential expression pattern of cytokine genes in three genetic groups showed different degree of mucosal immune response to *Eimeria* infection. In view of the relative expression of different cytokine genes, it can be concluded that Kadaknath birds were the most resistant to coccidial challenge followed by Cari-vishal and Cobb. These differences in expression of cytokine genes among the three genetic groups could be attributed to the fact that expression of cytokine genes varies depending upon the genetic background of birds, level of coccidial dosage, genotype of birds and age of infection. The mean relative fold expression of cytokine genes increased with dose and was lowest on day 14 post challenge. The relative expression of these cytokine genes could play a crucial role by driving higher immune responses susceptible breed/line.

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