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Comparative effects of inorganic and organic trace minerals (Zn, Se and Cr) supplementation on expression of ChTLR2b gene in broilers

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Abstract
Poultry is the fastest growing livestock sector in the developing countries. The global poultry sector is expected to continue to grow, as demand for poultry meat is driven by growing needs, population, rising incomes and urbanization. The present experiment was conducted to investigate the effect of inorganic and organic trace minerals supplementation on expression of ChTLR2b gene in broilers. A total of 216 broilers randomly divided into twelve groups and each group consisting of 18 broilers in 3 replicates. T1 group was kept as control. T_2, T_3 and T_4 group was supplemented with zinc (40 mg/kg of feed) inorganic, organic and 50% organic form respectively. T_5, T_6 and T_7 groups was supplemented with selenium (0.3 mg/kg of feed) from inorganic, organic and 50%organic form respectively. T_8, T_9 and T_{10} groups was supplemented with chromium (2 mg/kg of feed) from inorganic, organic form and 50% organic form respectively. T_{11} and T_{12} group was supplemented with combination of all 3 minerals from inorganic and organic form respectively. RT-PCR expression analysis of ChTLR2b gene in spleen revealed that maximum up regulation (3.8413 fold) was found in T_3 group (supplemented with organic Zn @ 40 mg/Kg of feed), followed by T_4 (3.5325 fold) whereas in bursa of fabricius the maximum up regulation (2.8921 fold) was found in T_6 group followed by T_{12} (2.5310 fold) as compared to control group. Up-regulation of gene expression ChTLR2b in bursa of fabricius and spleen indicates beneficial effect of organic trace minerals in potentiation of immune system in broilers.

Keywords: broilers, ChTLR2b gene, spleen, bursa of fabricius and organic trace minerals

Introduction
Broiler production in tropical countries is generally suboptimal as indicated by the poor growth performance and high mortality rate (Jaiswal *et al.,* 2017) [3].
Conventionally, inorganic trace minerals zinc (Zn), chromium (Cr) and selenium (Se) are used in chicken diet, because they are cost- effective and readily available, but are relatively inferior to organic trace minerals due to poor bioavailability (Virden *et al*., 2004) [10]. It is well established that organic trace minerals (OTMs) are environment- friendly because of their lower excretion rate and it remains long time in the gut consequently improves the growth performance (Leeson and Caston, 2008) [6]. Organic trace minerals (OTMs) have been used in the broiler industry in order to enhance the immune system to protect birds from the harmful effects of pathogenic microorganisms. Dietary immunomodulation has been introduced to the broiler industry as a strategy to control the pathogens and maintain the health of broilers (Yitbarek *et al*., 2012) [11]. Toll- like receptors (TLRs) which are types of pattern recognition receptors (PRR) are trans- membrane proteins expressed by cells of innate immunity as well as epithelial cells. Modulation of PRR expressed by cells of the innate immune system including macrophages and dendritic cells would be followed by production of cytokines IL-10 (Pragati *et al*., 2019) [9] some of which are involved in B cell development and antibody production (Hirayama *et al*., 2018) [2]. TLRs perform a vital role as sentinels of the innate immune system. TLRs also facilitate the development of adaptive immune responses. TLR2b are expressed in spleen and bursa (Kawasaki and Kawai, 2014) [5]. Therefore, in present study, use of TLR2b gene (immune regulatory genes) as a molecular marker to identify the immune-modulatory property of organic Zn, Cr and Se in poultry diets were studied.

Material and methods

The proposed research was carried out in the Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur (M.P.). A total of 216 broilers randomly divided into twelve groups and each group consisting of 18 broilers in 3 replicates. T_1 group was kept as control. T_2, T_3 and T_4 group was supplemented with zinc (40 mg/kg of feed) from inorganic, organic and 50% organic form respectively. T5, T6 and T_7 groups was supplemented with selenium (0.3 mg/kg of feed) from inorganic, organic and 50%organic form respectively. T_8, T_9 and T_{10} groups was supplemented with chromium (2 mg/kg of feed) from inorganic, organic form and 50%organic form respectively. T_{11} and T_{12} group was supplemented with combination of all 3 minerals from inorganic and organic form respectively.

Diets were formulated as per NRC (1994) [8] specifications. Inorganic and organic Zn, Se and Cr were supplemented along with feed as per the treatment groups. Broilers were kept in closed ventilated system for 35 days during the experimental period. Expression profile analysis of TLR2b in spleen and bursa of fabricius was done on day 35 of the experiment using RT-PCR technique. The recorded data was statistically analyzed using Completely Randomized Design. Various conditions and treatment groups were compared by using Duncan Multiple Range test (DMRT).

Total RNA was isolated from the bursa of fabricius and spleen following standard TRIzol method. The purity of RNA was checked before the preparation of first- strand cDNA. Prepared cDNA was stored frozen at -20 °C and was used for TLR2b gene expression studies. Expression of TLR2b gene was quantified using gene specific primer pairs using Real–Time PCR. β-actin was used as a reference gene.

RNA extraction

Isolation of RNA was done from aseptically collected tissue from broilers and these broilers were sacrificed following the appropriate standard procedure. TRIzol reagent (Sigma-aldrich, USA) was used to isolate total RNA from spleen and bursa of fabricius of broilers.

RNA quantification and DNase-1 treatment

The purity and concentration of the total RNA was assessed using Nanodrop Spectrophotometer (ND 1000, Thermo Scientific). The purity of the total RNA was confirmed by considering the ratios of OD values at 260 and 280nm between1.9-2.0. The integrity of RNA was checked on 1.0% agarose gel using 1x TBE as electrophoresis buffer.

The RNA samples showing contamination with DNA was incubated with RNase-free DNase-1 (MBI Fermentas) at 37 °C for 30 min. (@1 U for 1µg Total RNA). The DNase was subsequently inactivated by incubation at 65 °C for 10 min after adding the 25mM EDTA (@1 µl for 1µg Total RNA. Purity and concentration of DNase-treated total RNA sample was determined using nanodrop spectrophotometer.

First strand cDNA synthesis

The first strand cDNA was synthesized using Revert AidTM first strand cDNA synthesis kit (MBI Fermentas).

1. The components of kit were thawed at room temperature, mixed and briefly centrifuged and then stored on ice immediately.
2. A 20 µl reaction volume was used for 5 µg of total RNA. The following reagents were added into a nuclease free microcentrifuge tube on ice in the indicated order:

Table 1: The following reagents were added into a nuclease free microcentrifuge tube on ice in the indicated order

Reagent	Quantity
Total RNA	5 µg
Random Hexamer primer	1µl
Nuclease free water	To 12 µl
The obtained mixture was mixed gently and centrifuged briefly. Mixture incubated at 65 °C for 5 minutes and quick chilled on ice. The following components were added in indicated order:	
5X Reaction buffer	4 µl
RibolockRNase inhibitor (20 U/ µl)	1 µl
10 mMdNTP mix	2 µl
RevertAid M-MuL V RT (200 U/ µl)	1 µl
Total Volume	20 µl

3. The contents of the tube were mixed gently and centrifuged briefly and incubated for 5 minutes at 25 °C followed by 60 minutes at 42 °C.
4. The reaction was terminated by heating at 70 °C for 5 minutes.
5. The resultant cDNA was stored frozen at -20 °C till used.

Polymerase chain reaction (PCR) primers

Primers for TLR2b gene and β-actin (β-actin; used as housekeeping gene) were adopted from Echeverry *et al*. (2016) [1]. Sequence of gene specific primers for ChTLR2b gene and β-actin are as follows:

Table 2: Sequence of gene specific primers for TLR2b gene and β-actin are as follows

S. No	Gene	Primers	Annealing Temperature	Gene bank access
1	ChTLR2b	F: CGCTTAGGAGAGACAATCTGTGAA R: CTGTTTTAGGGATTTCAGAGAATTT	58 ºC	AJ009800
2	Chβ-actin	F:CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	61 ºC	X00182

Concentration optimization of cDNA and primers

To optimize the concentration of cDNA and primer, PCR was carried out with two fold serial dilution of cDNA (x, x/2, x/4, x/8 and x/16) and different primer concentrations i.e. 10, 5 and 2.5 pM.

PCR reaction mixture

Readymix Taq PCR Reaction mix with MgCl2 (Sigma Aldrich, U.S.A.) was used to prepare PCR reaction mixture. In a PCR tube 20 µl reaction mixtures was prepared as follows:

Table 3: In a PCR tube 20 µl reaction mixture was prepared as follows

Reagents	Quantity
2 X ReadyMixTaq PCR Reagent Mix	10 µl
Forward Primer (10 pM)	0.1 µl
Reverse Primer (10pM)	0.1 µl
cDNA	1 µl
Nuclease free water	8.8 µl
Total Volume	20µl

Mix gently and briefly centrifuge to collect all components to the bottom of the tube. The PCR tubes with all the components were then transferred to thermal cycler (Bio- Rad laboratories Inc. USA). The PCR protocol designed for 35 cycles is as follows and it was kept same for both the primers used:

Table 4: The PCR protocol designed for 35 cycles is as follows and it was kept same for both the primers used

S. N	Steps		ChTLR2b	β-actin
1.	Initial Denaturation	Temperature	94 °C	94 °C
		Time	10 min	10 min
2.	Denaturation	Temperature	94 °C	94 °C
		Time	1 min	1 min
3.	Annealing	Temperature	58 °C	60 °C
		Time	45 sec	45 sec
4.	Extension	Temperature	72 °C	72 °C
		Time	1 min	1 min
5.	Final Extension	Temperature	72 °C	72 °C
		Time	10 min	10 min
6.	Hold	Temperature	4 °C	4 °C
		Time	∞	∞

Agarose gel electrophoresis of PCR products

The PCR products were tested for amplification of specific gene by agarose gel electrophoresis using 2.0% agarose gel in 1x Tris Acetate EDTA Buffer (Sigma-Aldrich, U.S.A.). A total volume of 20 ml of 2.0% agarose (Sigma-Aldrich, U.S.A.) was prepared in 1x Tris Acetate EDTA Buffer and placed in microwave oven until melted. Molten agarose was allowed to cool to about 55 °C and ethidium bromide was added to give a final concentration of 0.5 µg/ml. The gel was poured on to electrophoresis trough fitted with comb. The gel was allowed to set on a flat surface for about 15 minutes. Electrophoresis trough was placed in an electrophoresis tank filled with 1x Tris Acetate EDTA Buffer. Samples were prepared on a parafilm by mixing 2 µl of Gel Loading Buffer (Sigma- Aldrich, U.S.A.) and 8 µl of PCR products were loaded in parallel with
100 bp ladder (Direct load PCR 100 bp low ladder, Sigma-Aldrich, U.S.A.). Electrophoresis was done at 70 volts for 10 minutes, then at 50 volts for 2 hour. Gel was viewed under a UV Trans-illuminator and photographed with gel documentation system (*BIO RAD* Gel Doc EZ Images) for future analysis.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR/Real Time PCR)

The relative expression of gene specific mRNA was quantified by qRT-PCR/Real-time PCR employing SYBR green chemistry using Real-time PCR system (CFX Connect Real-time System, Bio-Rad laboratories Inc. USA). All reactions were performed in nuclease-free 8 tube-strips with optically clear flat caps (Axygen Scientific, Inc. USA). For each sample a dissociation curve (melting curve) was generated after completion of amplification to ascertain the specificity of amplification. A negative control containing all the ingredients except cDNA template (Non-template control; NTC) was set up invariably for each master mix made for conducting the reactions. The results were expressed as CT values of target and reference genes in test (treatment) and control (calibrator) samples.

Table 5: Real time PCR reaction mixture

2X DyNAmo HS SYBR Green q PCR master mix	10 µl
Forward Primer (10 pM)	0.1 µl
Reverse Primer (10 pM)	0.1 µl
cDNA	1 µl
Nuclease free water	8.8 µl
Total Volume	20 µl

Real time PCR reaction protocol

PCR cycling conditions were: initial denaturation of 94 °C for 3 minutes, followed by 40 cycles of denaturation 94 °C for 30 seconds; annealing 58 °C for 30 seconds and extension 72 °C for 30 seconds.

Relative quantification

Comparative CT method (Livak and Schmittgen, 2001) [7] was used for relative expression of target gene in the test sample (treatment) relative to that of control sample (calibrator). The mRNA expression of target gene in test sample was expressed as "n- fold up/down regulation" in relation to control sample. For estimation of relative expression of target gene by the comparative CT method, CT values of target gene in test and control sample were adjusted to the CT values of a reference gene (endogenous/internal control). In the present study ChTLR2b was the target gene whereas β-actin was taken as reference gene. The CT for the target gene (ChTLR2b) and the CT for the reference gene (β-actin) was determined for each test sample and the control sample. The relative expression of target genes was estimated in term of fold change in mRNA expression, using the following formula:

Fold change in expression of target gene = $2^{-}\Delta\Delta CT$ where,
$\Delta\Delta$ CT = Δ CT test - ΔCT control/calibrator
Δ CT test = CT target gene - CT reference gene (In test / treatment group)
ΔCT control/calibrator = CT target gene - CT reference gene (In control/calibrator group)

Where,
CT target gene = mean of the cycle threshold (CT) value of the gene being tested CT reference gene = mean of the CT value of the housekeeping gene β-actin

Results and discussion

The mRNA expression levels of ChTLR2b gene on day 35, in spleen and bursa of fabricius sample of broiler birds has been presented in terms of fold change in expression in Table 06 and Figure 01. In all the samples maximum up regulation of ChTLR2b gene was found in bursa of fabricius. In the spleen samples, maximum up regulation was found in T5 (8.06 fold) followed by T9 and minimum up regulation was found in T2

(1.53 fold) group. In bursa of fabricius maximum up regulation was found in T9 (13.57 fold) and minimum up regulation was found in T8 (1.39 fold) group. Echeverry *et al.* (2016) [1] reported that organic trace mineral supplementation enhances local and systemic innate immune responses and modulates oxidative stress in broiler chickens. The gene expression analysis showed that OTM treatment resulted in no change in ChTLR2b expression among treatments, which is contrary to present findings. In the present investigation fold change expression of ChTLR2b gene was up regulated more in bursa of fabricius as compared to spleen, which might be explained by the fact that an additional proinflammatory response was induced by organic Se and blend of organic Zn, Cr and Se treatment groups. Expression of ChTLR2b, found in chickens normally occurs at an early stage of inflammation and acts as a chemo-attractant for chicken heterophils (Kaiser and Staheli, 2014) [4].

Table 6: Comparative gene expression profiling (fold change) of ChTLR2b gene in different treatment groups in spleen and bursa of fabricius in broiler

Gene Treatment	Bursa of fabricius	Spleen
T1	1.0000	1.0000
T2	1.6259	2.9956
T3	2.2514	3.8413
T4	1.8512	3.5325
T5	1.9251	2.9856
T6	2.8921	3.3351
T7	1.8114	2.3622
T8	1.8321	2.2515
T9	1.9147	2.4210
T10	1.7894	2.3324
T11	1.9143	2.6141
T12	2.5310	3.1127

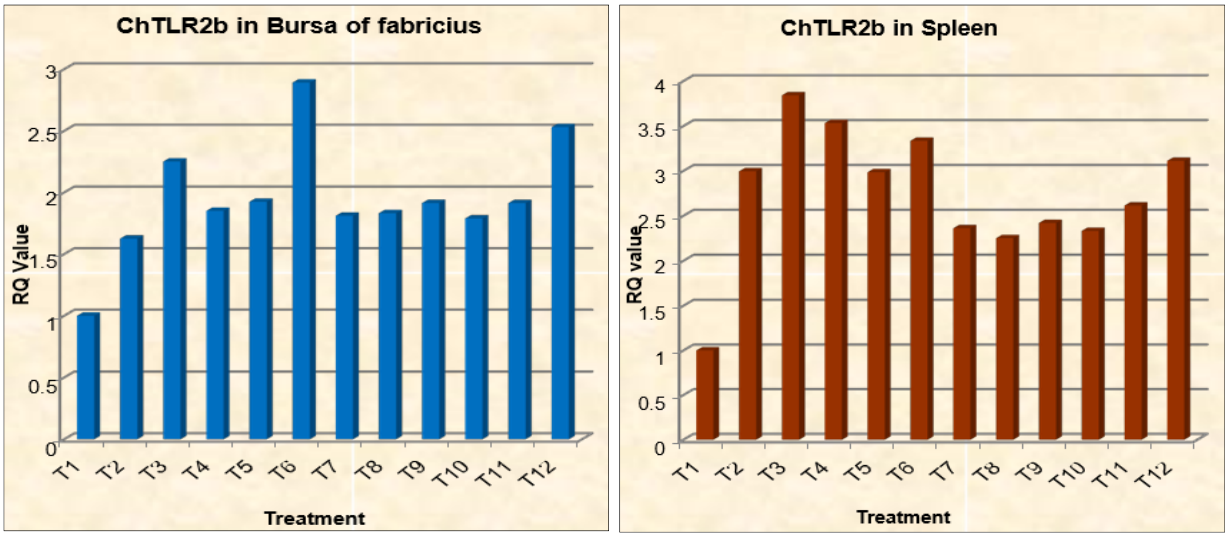

Fig 1: Comparative gene expression profiling (fold change) of ChTLR2b gene in different treatment groups in spleen and bursa of fabricius of broiler.

Conclusion

In all the samples maximum up regulation of ChTLR2b gene was found in bursa of fabricius. In the spleen samples, maximum up regulation was found in T5 (8.06 fold) followed by T9 and minimum up regulation was found in T2 (1.53 fold) group. In bursa of fabricius maximum up regulation was found in T9 (13.57 fold) and minimum up regulation was found in T8 (1.39 fold) group. Up-regulation of gene expression ChTLR2b in bursa of fabricius and spleen indicates beneficial effect of organic trace minerals in potentiation of immune system in broilers.

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