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Effect of organic trace mineral supplementation on expression of CxCLi2 gene in broilers

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Abstract
It is well recognized that demand of poultry meat and meat products has increased due to shortage of red meat supply and price phenomena. Annual broiler global meat production was 84.6 million tons in 2013 and covered 33% of global meat demand. It is well established that trace minerals are important for broiler normal growth and development. However, due to relative less economic importance, trace minerals sector failed to attract poultry scientist attentions. The current interest in trace minerals nutrition has been vigorously undertaken due to better bioavailability of trace minerals and concerns of environmental pollution. Hence, in the present investigation, the effect of organic trace minerals supplementation on expression of CxCLi2 gene in broilers was attempted. A total of 162 broiler birds randomly divided into nine groups were used for the experiment. Broilers were divided into 09 groups and each group consisting of 18 birds in 3 replicates. T1 group was kept as control. T2, T3 group was supplemented with zinc (40 mg/kg of feed) from inorganic and organic form respectively. T4, T5 group was supplemented with selenium (0.3 mg/kg of feed) from inorganic and organic form respectively. T6, T7 group was supplemented with chromium (2 mg/kg of feed) from inorganic and organic form respectively. T8, T9 group was supplemented with combination of all 3 minerals from inorganic and organic form respectively. RT-PCR expression analysis of CxCLi2 gene in spleen revealed that maximum up regulation (8.06 fold) was found in T5 group, followed by T9 (7.25 fold) whereas in bursa of fabricius the maximum up regulation (13.57 fold) was found in T9 group followed by T5 (10.10) fold as compared to control group.

Keywords: Broilers, CxCLi2 gene, organic trace minerals

Introduction

The genetic advancement has continuously elevated the broiler growth potential; broiler can attain mature body weight by consuming less feed and in shorter period of time. Trace minerals are indispensable components in the poultry diets. They are required for growth, bone development, well feathering, enzyme structure and function and appetite. Trace minerals act as catalysts for many biological reactions within the body.

In majority of tropical and subtropical countries, summer stress is severe, where the environmental temperature goes up to 45 °C and more. Higher ambient temperature is detrimental to body mass gain (Sahin, 2002) [5] and immune responses (Mashaly, 2004) [4] in young chickens. Heat stress increases production of corticosteroids, which suppress cell proliferation factor or interleukin-2 and interleukin-8 (CxCLi2) in broilers (Siegel, 1995) [6]. Therefore, in present study, use of some immune regulatory genes and cytokines 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 162 broiler birds randomly divided into nine groups were used for the experiment. Broilers were divided into 09 groups and each group consisting of 18 birds in 3 replicates. T1 group was kept as control. T2, T3 group was supplemented with zinc (40 mg/kg of feed) from inorganic and organic form respectively. T4, T5 group was supplemented with selenium (0.3 mg/kg of feed) from inorganic and organic form respectively. T6, T7 group was supplemented with chromium (2 mg/kg of feed) from inorganic and organic form respectively.

T8, T9 group was supplemented with combination of all 3 minerals from inorganic and organic form respectively.
Diets were formulated as per NRC specifications.Organic Zn, Se and Cr were supplemented along with feed. Broilers were kept in closed ventilated system for 35 days during the experimental period. Expression profile analysis of CxCLi2 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 CxCLi2 gene expression studies. Expression of CxCLi2 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 broiler and. These birds were sacrificed following the appropriate standard procedure. TRIzol reagent (Sigma-aldrich, USA) was used to isolate total RNA from liver of birds.

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 Aid™ 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:

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

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 CxCLi2 gene and β-actin (β-actin; used as housekeeping gene) were adopted from Echeverry *et al.* (2016) [1]. Sequence of gene specific primers for CxCLi2 gene and β-actin are as follows:

Sequence of gene specific primers for CxCLi2 gene and β-actin are as follows

S. No	Gene	Primers	Annealing Temperature	Gene bank access
1	CxCLi2	F: CCAAGCACACCTCTCTTCCA R: GCAAGGTAGGACGCTGGTAA	55 °C	AJ009800
2	Chβ-actin	F:CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	61 °C	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 $MgCl_2$ (Sigma Aldrich, U.S.A.) was used to prepare PCR reaction mixture. In a PCR tube 20 μl reaction mixture was prepared as follows:

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

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:

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

S. No.	Steps		CxCLi2	Chβ-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	58 °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 C_T values of target and reference genes in test (treatment) and control (calibrator) samples.

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 C_T method (Livak and Schmittgen, 2001) [3] 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 C_T method, C_T values of target gene in test and control sample were adjusted to the C_T values of a reference gene (endogenous/internal control). In the present study CxCLi2 was the target gene whereas β-actin was taken as reference gene. The C_T for the target gene (CxCLi2) and the C_T 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 = $2^{-\Delta\Delta C_T}$

where,

$\Delta\Delta C_T = \Delta C_{T\,test} - \Delta C_{T\,control/calibrator}$

$\Delta C_{T\,test} = C_{T\,target\,gene} - C_{T\,reference\,gene}$ (In test / treatment group)

$\Delta C_{T\,control/calibrator} = C_{T\,target\,gene} - C_{T\,reference\,gene}$ (In control/calibrator group)

where,

CT target gene = mean of the cycle threshold (CT) value of the gene being tested

CTreference gene = mean of the CT value of the housekeeping gene β-actin

Results and Discussion

The mRNA expression levels of CxCLi2 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 01 and Figure 01. In all the samples maximum up regulation of CxCLi2 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 CxCLi2 expression among treatments, which is contrary to present findings. In the present investigation fold change expression of CxCLi2 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. Secretion of CxCLi2, one of the 2 huCx- CLi8 orthologous found in chickens normally occurs at an early stage of inflammation and acts as a chemo-attractant for chicken heterophils (Kaiser and Staheli, 2014) [2].

Table 1: Comparative gene expression profiling (fold change) of CxCLi2gene in different treatment groups in spleen and bursa of fabricius in broilers

Treatment \ Organ	Spleen	Bursa of fabricius
T1	01.00	01.00
T2	01.53	04.44
T3	03.48	07.91
T4	02.92	03.65
T5	08.06	10.10
T6	04.83	05.71
T7	03.22	03.18
T8	02.20	02.18
T9	07.25	13.57

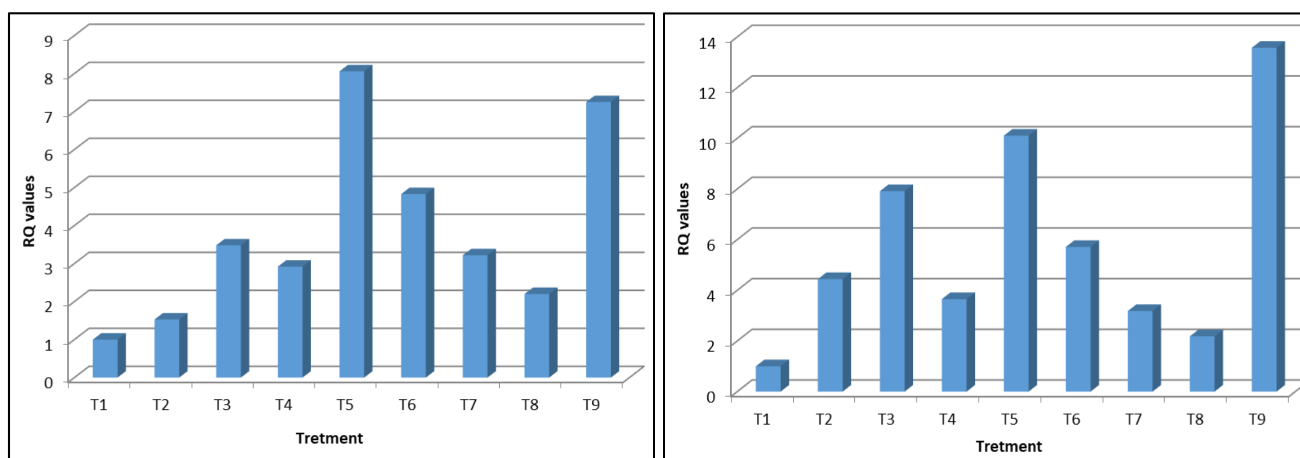

Fig 1: Comparative gene expression profiling (fold change) of CxCLi2 gene in different treatment groups in spleen and bursa of fabricius of experimental birds.

Conclusion

In all the samples maximum up regulation of CxCLi2 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 CxCLi2 gene expression in bursa of fabricius and spleen indicates beneficial effect of organic trace minerals in potentiation of immune system in broilers.

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