Original Research

Expression Profile of Cellular Retinol- Binding Protein IV (CRBP-IV) Gene in Rhode Island Red Chicken

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Abstract

The investigation was aimed to study expression profiling of Cellular Retinol- Binding Protein IV (CRBP-IV) in Rhode Island Red (RIR) chicken. Cellular Retinol Binding Protein IV (CRBP-IV) belongs to the family of cellular retinol binding proteins and plays a major role in absorption, transport, and metabolism of vitamin A. Vitamin A is correlated with reproductive performance, so measured relative mRNA expression of CRBP IV gene in kidney, liver and oviduct tissues collected from ten numbers of birds belonging to different ages viz., 12, 32 and 40 weeks, by quantitative reverse transcriptase PCR (qRT-PCR) method and data was analyzed using JMP of SAS (2010). Analysis revealed that the expression of CRBP IV gene differed significantly among three age groups in kidney ($p \leq 0.05$) and oviduct ($p \leq 0.1$) in RIR chicken. Highest expression of CRBP IV gene was observed at 40 weeks in kidney with a 40-ΔC_t value as 33.96±0.7 which was significantly more than those at 32 weeks and 12 weeks, where the estimates were 29.09±0.70 and 27.81±0.60, respectively. These data may help to understand the role of CRBP IV gene on the laying period of Rhode Island Red Chicken.

Key words: CRBP-IV Gene, mRNA Expression, RIR Chicken

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Introduction

The cellular retinol-binding proteins (CRBPs) are members of the intracellular lipid-binding protein (iLBP) multigene family. CRBPs are small molecular mass (~15 kDa) proteins that bind specifically to retinol and retinoic acid (Sundelin *et al*., 1985). CRBPs play important roles in intestinal vitamin A absorption and

cellular retinol transport due to the intense hydrophobicity of retinol and also control the metabolism and homeostasis of retinoids through interaction with metabolic enzymes (Ong, 1987, Giguere, 1994, Yin *et al*., 2014). There are four known CRBP genes, termed CRBP I, CRBP II, CRBP III and CRBP IV and CRBP IV play a role in absorption, transport, metabolism, and homeostasis of retinol and its derivatives. This makes CRBP IV a good candidate gene for enhancing reproductive traits in chickens (Yin *et al*., 2013). In India, poultry sector has shown a substantial improvement over the years and total egg production reached 82.93 billion with per capita availability of 66 eggs per annum (Singh *et al*., 2018). Rhode Island Red (RIR) chicken is useful for backyard poultry production and believed to be good egg producer. The basal mRNA expression profile in bird's different tissues are suggestive of its preparedness and performance ability of egg production. Scanty information available on basal expression level of CRBP IV gene in Rhode Island Red chicken. In view of the above, the present investigation was done to determine the basal mRNA expression level of CRBP IV gene in various tissues of RIR chicken.

Materials and Methods

Experimental Birds

A total of 225 straight run chicks were produced by mating of four RIR females each to eleven RIR males through Artificial Insemination. The birds were maintained at the experimental layer farm of this institute by providing *ad lib*. feed and water and following standard management and vaccination practices (Debnath *et al*., 2015). Ten pullets were selected randomly at different stages of laying i.e. four birds at pre-laying stage (12 weeks) , three birds at peak laying stage (32 weeks) and three birds at post-laying stage (> 40 weeks) for mRNA expression studies.

Sample Collection

Three tissues *viz.* liver, kidney and oviduct, weighing approximately 50-100 mg were aseptically collected from each of the ten experimental birds, in 2.0 ml centrifuge tube containing ~1.0 ml RNAlater® (Ambion, U.S.A.). Tissues were cut into small pieces to ensure proper infusion of RNAlater® into it and cryopreserved at -80ºC until used for RNA isolation. Sterilization of lab wares and inactivation of RNase was done by using 0.1% diethyl pyrocarbonate (DEPC) treated water and incubated at 37ºC for overnight.

Isolation of Total RNA

Total RNA from each tissue sample was isolated using TRIzol® reagent (Invitrogen, U.S.A) following step-wise procedure of Hongbao *et al*. (2008) and finally dissolved in 50 µl of nuclease- free water. Any possible contamination of genomic DNA was removed by 5 µl of each RNA sample with 5 U of RNase- free DNase (Biogene, USA) at 37ºC for 1 h. The DNase was subsequently inactivated by incubation at 65ºC for 10 min. RNA purity and quantity were determined by NanoDrop® (ND1000- Spectrophotometer) and samples

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showing absorbance ratio (260/280) of > 1.8-2 were considered to have satisfactory purity and used in subsequent analysis. The concentration of RNA was adjusted to 1000 ng/ µl before proceeding for synthesis of cDNA.

Synthesis of First Strand cDNA and Primers

One µl of total RNA from each sample was taken as template and first strand cDNA was prepared using Thermo Scientific Verso cDNA synthesis kit® (Thermo Fisher Scientific Inc., U.S.A.). The concentration of cDNA of each sample was equalized to 25 ng/µl for subsequent usage in qRT-PCR. Primer pairs of CRBP IV gene and housekeeping or the reference gene (β-actin) were selected from published literatures (Yin *et al*., 2013, Higgs *et al*., 2006) (Table 1). All the primers were synthesized by Xcelris Genomics Labs Ltd., Ahmedabad (India).

Table 1: Details of the primers for qRT-PCR

Target Gene	Primer Sequences	T_m (oC)	Amplicon Size (bp)	References
CRBP IV	F 5'- CATACCACAAGCACATTCAGAGA-3'	58ºC	125	Yin *et al*.(2013)
	R 5'- AGTTTGTCATTGTCCCAGGTAAC-3'			
β-actin	F 5'- GGA AGT TAC TCG CCT CTG -3'	58ºC	114	Higgs *et al*. (2006)
	R 5'- AAA GAC ACT TGT TGG GTT AC -3'			

Reaction Mixture

All PCR reactions were carried out in triplicate in 0.2 ml clear, thin walled nuclease-free 8-tube strips with optically clear flat lid (Axygen Scientific Inc., U.S.A) to avoid pipetting error. A negative control (NTC, no template control) in triplicate containing all the ingredients except the template (cDNA) was also set up to check any contamination. β-actin gene was used as reference gene. The amplification was performed in 20 µl reaction mixture using DyNAmo ColorFlash SYBR Green qPCR Kit® (Thermo Fisher Scientific Inc., U.S.A.).

Real Time-PCR Programme, Retrieval Compilation of qRT-PCR Data

Relative mRNA expression of CRBP IV gene in each of the three tissues *viz.* liver, kidney and oviduct was done by quantitative reverse transcriptase PCR (qRT-PCR) in CFX 96® in Real Time PCR detection system (Bio-Rad Laboratories Inc., U.S.A.) and Real-time PCR cycling conditions used were as follows: initial denaturation at 95ºC for 7 minutes followed by 40 cycles of denaturation at 95ºC for 10 seconds, annealing at optimized temperature for 20 seconds and extension at 72ºC for 20 seconds; followed by detection of fluorescent signal by the real time detection system to generate amplification curve. After completion of 40 cycles, each sample was subjected to 60-95ºC @ ±0.5ºC increment for 10 seconds to generate dissociation curve or melt curve to identify specific amplification. Afterwards, threshold cycle (C_t) value and melting point temperature of each tube was retrieved and reviewed for its corresponding amplification and

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dissociation curve to ensure appropriateness of specific amplifications. Subsequently, data were imported into MS-Excel file and saved for further statistical analysis.

Determination of 40-ΔC_t and Fold Expression of Genes by $2^{(-\Delta\Delta Ct)}$ Method

The tissue showing highest ΔC_t value was chosen as the calibrator tissue. For each sample, the ΔC_t value was subtracted from 40 (total cycle number) so as to obtain 40-ΔC_t. Higher 40-ΔC_t value was considered as higher expression (MacKinnon *et al.*, 2009). The fold change expression was determined by using the formula $2^{(-\Delta\Delta Ct)}$ method as per Livak and Schmittgen (2001). The standard errors of ΔC_t values were calculated for comparison of the three tissues at different age groups (Yuan *et al*., 2006).

Statistical Analysis of qRT-PCR Data

Differential expression of target gene in the three tissues at three different ages of RIR pullets was analyzed by least squares analysis of variance (LS ANOVA) using JMP 9.0.0 statistical program package (SAS, 2010). Hatch and age was taken as fixed effect in the model.

$$Y_{ijk} = \mu + A_i + H_j + e_{ijk}$$

Where,

Y_{ijk} = 40-ΔC_t value of mRNA expression of gene under study in i^{th} age, j^{th} hatch and k^{th} tissue of individual pullet

μ = overall mean

A_i = fixed effect of i^{th} age of individual pullet (i = 1, 2 and3)

H_j = fixed effect of j^{th} hatch (i = 1, 2)

e_{ijk} = random error associated with k^{th} individual pullet of i^{th} age in j^{th} hatch (mean '0'; variance 'σ^2')

Results and Discussion

The least squares means revealed that age had significant effect on expression of mRNA of CRBP IV gene in kidney, liver and oviduct of RIR chicken (Table 2).

Table 2: Least squares analysis of variance of relative mRNA expression levels (40-ΔC_t values) of CRBP IV gene indifferent tissues at various ages in RIR chicken

Source of Variation	Df	Kidney		Liver		Oviduct	
		MSS	p value	MSS	p value	MSS	p value
Age	2	33.12948	0.0015**	8.414685	0.2393	5.718642	0.0979#
Hatch	1	7.177953	0.0657	1.436986	0.5962	1.236144	0.4173
Remainder	6	1.4211					

*MSS: mean sum of squares, #p≤0.1, **p≤0.01, df: denotes degrees of freedom*

The expression of CRBP IV gene (mean 40-ΔC_t values) differed significantly among three age groups in kidney ($p \leq 0.05$) and oviduct ($p < 0.1$) in RIR chicken. The 40-ΔC_t value at 40 weeks in kidney was 33.96±0.7 which was significantly higher than those at 32 weeks (29.09±0.70) and 12 weeks (27.81±0.60). In oviduct,

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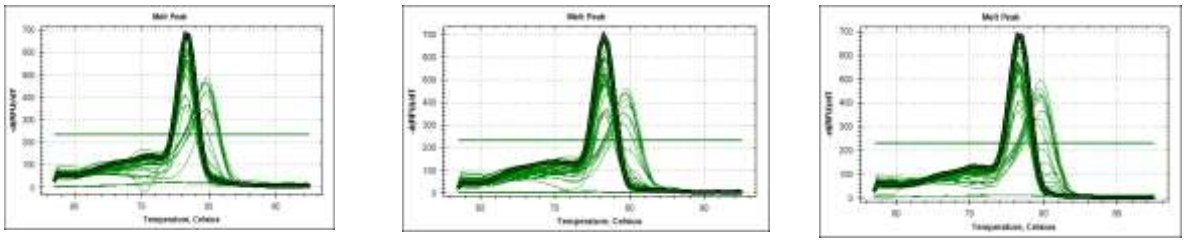
the highest relative mRNA expression of CRBP-IV gene was seen at 40 weeks (30.95±0.75).But in liver, the mRNA expression did not differ significantly ($p>0.05$) among three ages. Analysis also revealed that highest expression (30.29±0.38) was observed in kidney tissue, followed by oviduct (29.36±0.41) and liver (28.85±0.68) tissues (Table 3).

Table 3: Least squares mean ± standard error of 40-ΔC_t values of mRNA expression levels of CRBP IV gene in different tissues at various ages in RIR chicken

Factors	N	Kidney	Liver	Oviduct
Over all	**10**	30.29±0.38	28.85±0.68	29.36±0.41
Age of RIR chicken in Weeks				
12	4	27.81±0.60^b	30.33±1.07	28.89±0.64^b
32	3	29.09±0.70^b	29.07±1.26	28.25±0.75^b
40	3	33.96±0.70^a	27.17±1.26	30.95±0.75^a
Hatches				
1	5	29.41±0.54	28.46±0.98	29.00±0.58
2	5	31.17±0.54	29.25±0.98	29.73±0.58

N: Number of observations; Means with different superscripts in a column differ significantly.

Previously, Yin *et al*. (2013) studied the mRNA expression in Erlang Mountainous chickens and reported that the CRBP IV mRNA levels changed with age in the various tissues at different ages (12, 24, 32 and 45 weeks) and also reported that high expression was found in all tissues at 32 weeks, except for the heart and low expression was found at 12 weeks and 45 weeks as vitamin A is highly required during laying time and concluded that CRBP IV may play an important role in vitamin A metabolism. In present study, it was seen that expression of CRBP IV gene was tissue- specific and highly expressed at the age of egg production. High expression in kidney might be due to vitamin A metabolism being regulated by kidneys as Yin *et al*. (2013) reported the same in Erlang Mountainous chickens. In our present investigation CRBP-IV was highly expressed at a later age of 40 weeks. Differences in reports might be due to the differences in the germplasm analyzed. Further, melting curve analysis demonstrated a single predominant peak with a distinct melting temperature for the primer pairs (Fig. 1).



(A) **(B)** **(C)**

Fig. 1: Melting (A, B & C) curves of CRBP-IV gene mRNA during qRT-PCR in Kidney, Liver and Oviduct tissues, respectively in pure strain of RIR chicken

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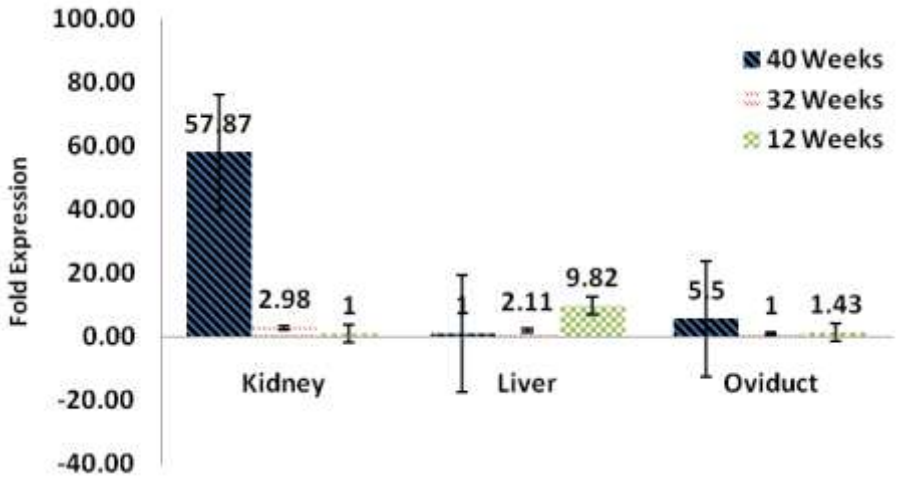

Fig. 2: Fold expression of CRBP IV gene at various ages in kidney, liver and oviduct of pure strain of RIR chicken

Fold changes of relative mRNA expression of CRBP IV gene in different tissues (kidney, oviduct and liver) of RIR chicken are presented in Fig. 2. It was found that CRBP IV gene expressed 57.87 folds more at 40 weeks of age in kidney than at 12 weeks. The expression at 32 weeks was 2.98 folds more than at 12 weeks. In oviduct, CRBP IV gene expressed 5.5 folds more at 40 weeks than 32 weeks. It was 1.43 folds more expressed at 12 weeks of age than at 32 weeks of age. In liver, CRBP IV gene expressed 9.82 folds more at 12 weeks than at 40 weeks. The expression at 32 weeks was as 2.11 folds more than at 40 weeks of age in liver. However, reports on comparison of basal mRNA expression levels across tissues in RIR and other chicken germplasm were not available in the literature. Thus, it can only be concluded that kidney and oviduct tissues revealed maximum fold expression at the age of 40 weeks of the gene studied.

Conclusion

In view of the above findings, it may be inferred that the basal mRNA expression level of CRBP IV gene in RIR chickens was tissue specific and showed egg laying time- dependent changes. Thus, the results suggest that CRBP IV may play an important role in egg production traits in Rhode Island Red chicken.

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References

1. Debnath, J., Kumar, S., Bhanja, S. K., Rahim, A., & Yadav R. (2015). Factors influencing early layer economic traits in Rhode Island Red chicken. *Journal of Animal Research*, 5(4), 915–19.
2. Giguere V. (1994). Retinoic acid receptors and cellular retinoid binding proteins: Complex interplay in retinoid signaling. *Endocrine Reviews*, 15, 61-79.
3. Higgs, R., Cormican, P., Cahalane, S., Allan, B., Llod, A. T., Meade, K., James, T., Lynn, D. J., Babiuk, L. A., & O'Farrelly, C. (2006). Induction of a novel chicken toll-like receptor following Salmonella enteric serovar Typhimurium infection. *Infection and Immunity, 74, 1692*-1698.
4. Hongbao, M., Young, J., & Shen, C. (2008). RNA, DNA and protein isolation using TRIzol reagent. *Nature and Science*, 6(3), ISSN 1545-0740.
5. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using realtime quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ method. *Methods*, 25, 402-408.
6. MacKinnon, K. M., He, H., Nerren, J. R., Swaggerty, C. L., Genovese, K. J., & Kogut, M. H. (2009). Expression profile of toll-like receptors within the gastrointestinal tract of 2-day-old Salmonella enteritidis infected broiler chickens. *Veterinary Microbiology*, 137, 313-319.
7. Ong, D. E. (1987). Cellular retinoid-binding proteins. *Archives Dermatology,* 123,1693-1695.
8. SAS. (2010). SAS Institute Inc., SAS Campus Drive, Cary, NC, USA 27513.
9. Sinha, B., Mandal, K., Kumari, R., & Kumari, T. (2018). Estimate and Effect of Breeds on Egg Quality Traits of Poultry - A Review. *International Journal of Livestock Research*, 8(4), 8-21. http://dx.doi.org/10.5455/ijlr.20170812102444.
10. Sundelin, J., Eriksson, U., Melhus, H., Nilsson, M., Lundvall, J., Båvik, C. O., Hansson, E., Laurent, B., & Peterson, P. A. (1985). Cellular retinoid binding proteins. *Chemistry and Physics of Lipids*, 38, 175-185.
11. Yin, H. D., Tian, K., Li, D. Y., Gilbert, L. H., Xiao, L. H., Chen, S.Y., Wang, Y., Liu, Y.P., Zhao, X. L. & Zhu, Q. (2014) . Expression profiles of cellular retinol-binding protein type II (CRBP-II) in Erlang Mountainous chicken. *Asian-Australians Journal of Animal Sciences*, 27 (3), 310-315.
12. Yin, H. D., Wang, Y., Zhang, Z. C., Liu, Y. P., Chen, S. Y., & Zhu, Q.(2013). Characterization of the expression profile and genetic polymorphism of the Cellular Retinol-Binding Protein (CRBP-IV) gene in Erlang Mountainous chickens. *International Journal of Molecular Sciences*,14, 4432-4443.
13. Yuan, J. S., Reed, A., Chen, F., & Stewart Jr, C. N. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics*, 7,85.Doi: 10.1 186/147-2105-7-

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