



## Tissue-specific expression of troponin C (*TNNC1*) gene in indigenous native chicken

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The contractile proteins are commonly expressed in a tissue specific manner (Parmacek *et al.* 1994). The striated muscle contraction is regulated by troponin complex and tropomyosin located in the thin filament of the myofibrils. Troponin C is a calcium binding subunit of the troponin complex (Hanson *et al.* 1973, Ohtsuki 1975) which modulates the contraction of striated muscles in association with tropomyosin and other troponin (T and I) components (Ebashi *et al.* 1971, Weber and Murray 1973). Besides, 2 different isoforms of troponin C were identified, such as troponin C of cardiac and slow skeletal muscles, which have descended from a single gene (Dhoot *et al.* 1979, Wilkinson 1980). The chicken cardiac troponin T gene exhibits both tissue-specific and temporal transcriptional regulation during development. However, the present investigation demonstrates the expression profile of troponin C mRNA in different tissues at different age groups in two different indigenous breeds of chicken.

The present investigation was carried out in 2 slow growing indigenous chicken populations, namely Aseel and Ghagus, maintained at the institute. All the birds were reared on deep litter system in the same management with *ad lib.* feeding and watering. Further, all the birds were hatched at the same time and housed all along in the same shed. Identical environmental conditions were maintained throughout the experimental period to nullify the effect of environment.

Expression profile was studied in 5 different tissues, viz. such as muscle, bursa, heart, spleen and gizzard tissues on 1<sup>st</sup> and 28<sup>th</sup> day of age. All the tissue samples (2 samples

from each sex, each breed and each respective day) were collected under aseptic conditions following the approved slaughtered protocol of Institutional Animal Ethics Committee (IAEC). The tissues were collected into 1.5 ml DNase RNase free sterile polypropylene tubes under aseptic conditions by using DEPC treated sterile instruments. Samples were immediately chilled on ice and stored at  $-80^{\circ}\text{C}$  until RNA extraction. All the tissue samples were homogenized in RNA isolation reagent Trizol (Invitrogen) according to manufacturer's instructions. Thus, RNA pellet was re-suspended in 50  $\mu\text{l}$  of nuclease-free water. The isolated RNA samples were treated with DNaseI for removal of possible genomic DNA contamination. The quality of RNA was determined by the  $A_{260/280}$  absorbance ratio and the samples having the OD value of 1.6 to 1.9 were considered of good quality. The integrity of the 18S and 28S rRNA bands was checked by 2.2 M formaldehyde denatured agarose gel electrophoresis. Further, High Capacity cDNA Reverse Transcription kit (Applied Biosystems<sup>TM</sup>) was used for the synthesis of cDNA from isolated RNA samples. The reaction was performed in a volume of 20  $\mu\text{l}$  containing 10 $\times$  RT Buffer (2.0  $\mu\text{l}$ ), 10 $\times$  RT random primers (2.0  $\mu\text{l}$ ), 100 mM of each dNTPs (0.8  $\mu\text{l}$ ), RNase inhibitor (1.0  $\mu\text{l}$ ), 50U MultiScribe Reverse Transcriptase (1.0  $\mu\text{l}$ ), Nuclease-free water (3.2  $\mu\text{l}$ ), and the template RNA (10  $\mu\text{l}$ ). The reverse transcription was performed at 25 $^{\circ}\text{C}$  for 10 min, 37 $^{\circ}\text{C}$  for 120 min, 85 $^{\circ}\text{C}$  for 5 min, and terminated with 4 $^{\circ}\text{C}$  for 5 min, then products were stored at  $-20^{\circ}\text{C}$ .

The real time quantitative PCR using SYBR green (Maxima SYBR Green/ROX qPCR Master Mix, ThermoFisher Scientific) was performed to know the expression levels of target (*TNNC1*) and reference (*GAPDH* and  $\beta$ -*actin*) genes. For normalizing different amounts of input RNA, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and  $\beta$ -*actin* genes were used as internal control. A pair of intron spanning primers (Table 1) for each respective gene was designed by using Primer-Blast (synthesized from Sigma Aldrich, Bengaluru). All the samples were analyzed in duplicates with a final volume of

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10 µl comprising 5 µl of 2× SYBR Green/ROX qPCR master mix, 1 µl of each forward and reverse primer (20 ng), 1 µl of template cDNA and 3 µl of nuclease free water. The thermal cycling conditions followed were initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 sec, primer annealing at 58°C for 30 sec and extension at 72°C for 30 sec. Melt curve analysis (95°C for 15 sec and 60°C for 1 min) was performed at the end of qPCR to check the specificity of amplification.

The quantification cycle (Cq) or threshold cycle (Ct) value of the target and the reference genes were determined for all qPCR reactions. The mRNA expression of target gene was analyzed by comparative Ct method of relative quantification. The gene quantification was expressed as n-fold up/down regulation of transcription, in relation to an internal control. The expression of target gene was calibrated by that of the reference gene, at each time point and converted to the relative expression (fold of expression), as follows:

$$\text{Fold of expression} = 2^{-\Delta\Delta Ct}$$

where  $\Delta Ct$ , average Ct of target gene – average Ct of reference gene (*GAPDH* and  $\beta$ -actin);  $\Delta\Delta Ct$  = average  $\Delta Ct$  of target sample – average  $\Delta Ct$  of calibrator sample.

The raw fluorescence data of both target and reference genes were subjected to logarithmic transformation for further statistical analysis. Statistical analysis was carried out using SPSS 20 version. ANOVA test was performed to compare the mRNA expression levels among different tissues. In addition, student 't' test was performed to compare the expression levels between different breeds and age groups.

Each pair of primers used in the experiment was checked for amplification efficiency by step dilutions. In addition, the template cDNA was tenfold serial diluted and average Cq value was recorded at each dilution for both target and reference genes. The relative standard curve (Fig. 1) was plotted by drawing the cDNA dilutions (independent variable) in logarithmic scale on X-axis and quantification cycle (dependent variable) on Y-axis. The curve determined regression coefficient (slope) as -3.466, -3.408 and -3.352 for *TNNC1*, *GAPDH* and  $\beta$ -actin genes, respectively. However, the coefficient of determination ( $R^2$ ) was 0.9883, 0.9989 and 0.9925 for *TNNC1*, *GAPDH* and  $\beta$ -actin genes, respectively. Statistical analysis found regression coefficient

as significant ( $P < 0.05$ ) for both target and reference genes representing that standardization of gene expression was perfected before continuing to the proper experiment.

The expression of *TNNC1* mRNA was detected in all the tissues analyzed in this study. Irrespective of breed, relative to the reference genes, the mRNA expression levels of the target gene varied considerably in different tissues. At day old age, the *TNNC1* transcript had a relatively higher expression in heart (42.17), followed by spleen (39.30), muscle (37.51), bursa (33.09) and gizzard (31.81). At day 28, the highest magnitude of expression was found in heart (41.33), followed by muscle (39.68), spleen (34.98), bursa (33.86) and gizzard (31.42). Overall, a significant ( $P < 0.05$ ) difference in the expression of the *TNNC1* gene was noticed among all the tissues studied. When compared to the expression level in gizzard, the gene expression was upregulated in heart by 1314.17 and 962.85 folds; in muscle by 51.96 and 307.23 folds; in spleen by 179.30 and 11.80 folds and in bursa by 2.43 and 5.41 folds, at day 1 and 28 of age respectively. Furthermore, Toyota *et al.* (1989) also demonstrated the expression of troponin C in heart (atrial and ventricular muscles) and skeletal muscles of chicken during embryonic period. As the heart contains highly contractile smooth muscles, the higher expression level of target gene was observed in cardiac muscles. Sex had no significant effect on the expression of the target gene. Stoutamyer and Dhoot (2005) reported the transcripts of the target gene are not only expressed in quail embryonic

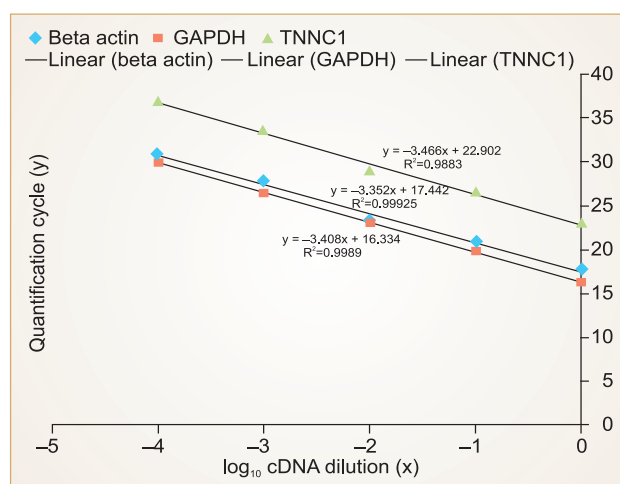


Fig. 1. Standard curve for optimizing realtime PCR with respect to *TNNC1*, *GAPDH* and  $\beta$ -actin genes.

Table 1. Primer used for amplification of genes

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Annealing temp (°C)	NCBI Accession Number
<i>TNNC1</i>	F: TGCCTTCGACATCTTCGTGCTG R: GCCAGCAATCTGCCATCCTCCT	145	58	NM_205133.1
<i>GAPDH</i>	F: CTGCCGTCCTCTCTGGC R: GACAGTGCCCTTGAAGTG	119	58	NM_204305
$\beta$ -actin	F: TGGACTCTGGTGAATGGTGT R: GTGGTGAAGCTGTAGCCTCT	148	58	NC_006101.4

skeletal muscle but also in embryonic heart.

The developmental changes of the *TNNC1* mRNA expression in chickens at day-old and day-28 age groups were also analyzed. Irrespective of breed, the magnitude of expression at day-old was 37.51, 33.09, 42.17, 39.30 and 31.81 and at day-28 was 39.68, 33.86, 41.33, 34.98 and 31.42 in muscle, bursa, heart, spleen and gizzard, respectively. Further, the expression levels differed significantly ( $P < 0.05$ ) between these age groups in spleen and muscle tissues. In muscle, as compared to expression at day-old, 4.52 fold of up regulation was observed at 28-day of age. In spleen, 19.87 fold upregulation of expression was found at day-28 as compared to 2 day-old age group. Further, no significant difference was found in mRNA expression levels in heart, bursa and gizzard between different age groups.

Within breeds, in Ghagus, a significant ( $P < 0.05$ ) difference in mRNA expression was noticed in spleen between different age groups, where, it showed 39.31 fold of higher expression at day-old (40.49) in relation to day-28 (35.19). In Aseel, we found a significant ( $P < 0.05$ ) difference in the magnitude of expression in muscle tissue between different age groups where, 10.66 fold of upregulation was seen in day-28 (40.72) when compared with day-old (37.31).

In Ghagus birds, the level of expression at day-old was 37.71, 33.44, 41.17, 40.49 and 33.43 whereas, at day-28 it was 38.64, 33.95, 39.98, 35.19 and 31.41 in muscle, bursa, heart, spleen and gizzard, respectively. The respective values in Aseel birds were 37.31, 32.74, 43.17, 38.10 and 301.9 at day old and 40.72, 33.76, 42.68, 34.77 and 31.43 at day 28. No significant difference in expression was found between Ghagus and Aseel breeds at day 1 and day-28.

In conclusion, we explored the tissue distribution in terms of expression levels of the *TNNC1* mRNA in chicken. The expression of *TNNC1* was tissue specific and varied considerably among different tissues at different age groups. Among all the tissues, the highest magnitude of target gene expression was observed in heart at day old as well as at 28<sup>th</sup> day. All these results showed that the expression of *TNNC1* mRNA varied between age groups and tissues.

## SUMMARY

Troponin C is a component in troponin complex and participates in the contraction of smooth muscles. The present research investigated the expression of troponin C type 1 (*TNNC1*) in different tissues at different age groups in Ghagus and Aseel, the indigenous breeds of chicken population. A significant difference in expression level of *TNNC1* mRNA was noticed among various tissues. In spleen and muscle tissues, the expression level varied significantly between day-old and day-28. With respect to different breeds, no significant difference was found in the level of expression at day 1 and day 28. In conclusion, it is stated that the expression of *TNNC1* mRNA varied between tissues and age groups in chicken.

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