The Editor
International Journal of Agriculture and Biology.

 Dear Sir/Madam

We feel immense pleasure to submit our research article entitled “**Potential effects of CEMB Bt corn on immunology and hormonal metabolism in Broiler chicken**” to be considered for the peer review process in your prestigious journal “International Journal of Agriculture and Biology”.

GM crop has been cultivated around the globe for last two decades. Risks related to GM crops are also debatable. CEMB developed the first-ever Bt corn containing cry1Ac+cry2A genes that have insecticidal activity. Before commercializing the GM crop biosafety assessment of the Bt-corn is mandatory. Corn is the major ingredient in the poultry feed therefore broiler chickens were selected to assess the potential toxic effects of the Bt-corn through feeding along with commercial diet. A feeding trial was conducted for 45 days after results of the biochemical and genes expression via qPCR (Immune and Growth related genes) of the experimental animals revealed no any significant toxic effects. The promising results of this feeding trial recommended that the CEMB-Bt corn is bio safe and can be used in poultry feed.

 We strongly believe that the research data will be interesting for the scientific community. Besides that, we also believe that the article has been prepared well according to the author guidelines of the journal and has not been submitted else for peer review process except in “International Journal of Agriculture and Biology”.

Sincerely yours,

Muhammad Tariq

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**Title page**

**Title: Potential effects of CEMB Bt corn on immunology and hormonal metabolism in Broiler chicken**

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**Novelty Statement**

Broiler chickens were selected for the biosafety studies of CEMB Bt-Corn. This is first ever Bt corn developed in Pakistan by CEMB, University of the Punjab. Potential risk associated with this Bt-corn were assessed after feeding to the developing broiler chickens. Gene expression of the immune and growth-related genes in chickens has been reported in this study after feeding on GM corn diet. The present study provides interesting insights into the risk assessment of GM corn diet.

**Potential effects of CEMB Bt corn on immunology and hormonal metabolism in Broiler chicken**

**Abstract**

Genetically modified crops utilize commercially throughout the world, thus require comprehensive biosafety assessment study before commercial releasing. For this purpose many studies have been performed on different species of animals to examine unintended toxic effects. Current study was to analyze the potential toxic effects of CEMB-Bt corn containing insecticidal *Bt* genes c*ry1Ac/2A* developed by CEMB on broiler chickens through feeding. A total of 60 model animals procured, vaccinated and randomly divided into four groups. Control group fed 0 % while experimental groups fed 30 %, 40 % and 50 % GM corn along with the commercial diet for 45 days. The nutritional analyses of diet fed to all four chicken groups revealed no significant difference. The total Bt proteins with maximum concentration was 1.62 µg/gram of the diet was observed in the diet of experimental group containing 50 % GM corn. Animals were sacrificed and sampled for biochemical and molecular investigations. Biochemical results revealed that no significant alteration in the biochemical parameters among control and experimental groups. Expression of growth and immune response genes were analyzed through qPCR. No significant effects on the relative expression of growth and immune related genes were observed except *Growth Hormone* gene expression of control group is slightly low and significant different to the transgenic groups. However, in conclusion consumption of GM Bt corn seeds did not induce any effects on growth and health of growing broiler chickens.

**Key words**: Genetically modified, food security, chicken feed, CEMB-Bt corn, growth and immune related genes.

**Introduction**

GM crops progressed the agriculture by improving the crops productivity to ensure an adequate food supply, enhanced nutritional quality, taste, herbicide, pests and diseases resistance, increased shelf life along with abiotic stress resistance (Meiri and Altman, 1998). In 1983, first GM plant (*Nicotiana tabacum)* was developed with antibiotic resistance (Woolsey, 2012). Currently, GM crops are being cultivated on an area of approximately 190 million hectares in the world with GM cotton, corn and soybean being the prominent (Turnbull et al., 2021). Numerous studies in past have documented an expansion in development of the R&D sector for GM crops (Graff et al., 2009; Miller and Bradford, 2010). About 93.3% crops (corn, cotton and soya) cultivated in USA are biotech varieties (Baghbani-Arani et al., 2021) and more than 70% of the processed food products are genetically engineered in Canada (Mitchell, 2002). To reduce the fear of hunger and poverty, developing countries are also adopting genetic engineering techniques. In these countries, 40% of the total farming area used for GM crop cultivation (Malik and Ahsan, 2016). The GM corn has more approved events than any other transgenic crop. It was planted around the globe at 53.6 million hectares in 2015 which was about one-third of the total cultivated area. Out of these 53.6 million hectares, 33 million hectares were planted in the United States and 17.4 million hectares were cultivated in Canada, Brazil, and Argentina. The global net worth of transgenic corn is 8.1 billion US dollars (Pellegrino et al., 2018).

After wheat and rice, the third primary cereal crop of the world is the corn and commercially cultivated around the globe. In the year 2021, the cultivation area and production of the corn increased in 16.6 % and 19.0 % respectively in Pakistan (Economy Survey of Pakistan, 2022). Only transgenic crop cultivated in Pakistan is cotton. But in the future, to secure from the sudden outbreak of insects infestation in the corn crop, Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Pakistan has developed a Bt transgenic corn inbred line expressing two Cry toxin (Cry1Ac & Cry2A) (Farooq, 2017) and is in pipeline for the commercialization. Risk assessment of any developed GM crop is mandatory pre-requisite for commercialization. Possible risks associated with the GM food could be allergenicity (Gabol et al., 2012), harm to other non-targeted organisms (Losey et al., 1999), adverse health effects on the experimental animals (Sánchez and Parrott, 2017) and any hematological, immunologic and biochemical effects (Dona and Arvanitoyannis, 2009). Inconsistent reports and contradictory opinions regarding the possible dangers of GM crops to human health further sensitize the risks associated with GM crops. Moreover, not enough information is available regarding the legitimacy of safety assessment tests that were carried out for these crops (Ibrahim and Okasha, 2016). Corn is used in poultry feed (Anami and Widanarti, 2020), consumption of total annual grain by Pakistan commercial poultry industry is estimated at 4.23 million tons out of which 2.42 million tons (57%) is met from corn (Habib et al., 2016). Hence, Biosafety study was designed on broiler chicken being fed with CEMB-Bt corn in varied concentrations to reveal any potential risk associated with the CEMB-Bt corn.

Broiler chicken’s ability to quickly gain weight makes them highly responsive to any modification or toxins in diet so, broiler chicks were selected for this study. In this study the potential toxic effects of CEMB-Bt corn was evaluated by feeding the developing broiler chicken by assessing the growth pattern, biochemical analysis and effects of growth and immune related genes expression.

**MATERIALS AND METHOD**

**Test Material**

The test substance was genetically modified corn variety, CEMB-Bt corn. Two insecticidal Cry toxin genes (c*ry1A*c+c*ry2A*) derived from *Bacillus thuringenesis* and modified for enhanced expression were integrated in CEMB inbred corn lines. The illustration of the binary construct harboring *cry* genes is depicted as Figure 1.

**Confirmation of *Bt* gene insertion in CEMB corn inbred lines**

The insecticidal Cry toxin genes; c*ry1Ac* and *cry2A* were expressed as a single T-DNA insertion in the transgenic, hence transgene insertion could be achieved either through *cry1Ac* gene amplification or *cry2A* (Lee and Gelvin, 2008) . For this, genomic DNA (gDNA) was extracted from the seeds of GM and non-GM corn samples through the modified CTAB method. A 2X CTAB buffer (2 % CTAB, 1 % PVP , 1.4 M NaCl, 100 mM Tris HCl pH 8.0, 20 mM EDTA pH 8.0 and H2O) and extraction Buffer (20mM Tris HCl pH 8.0, 25mM EDTA pH 8.0, 200mM NaCl, 0.5 % SDS and H2O) was heated at 65℃ in the water bath prior to extraction. 10-100 mg seed sample was ground in 600 μl pre-warm extraction buffer. The mixture was transferred to an eppendorf tube and 400 μl of 2X CTAB buffer along with 5μl of β-Mercapto-Ethanol was added and the samples were vortexed for 1 to 2 min. Then samples were incubated at 65oC in the water bath for 1 hour accompanied by occasional shaking. Samples were cooled down on the ice for 5 min and added the 400μl of chilled Chloroform:Isoamyl alcohol (24:1). Later, the samples were vigorously shaked and centrifuged at 13000 rpm for 20 min at 4oC. The supernatant was shifted to a new tube and 2/3 volume of ice cold isopropanol was added and mixed by several inversions of tube gently. The samples were incubated for 1 hour at -20oC and centrifuged at 13000rpm for 15 min at 4oC. The supernatant was removed and the pellet (gnomic DNA) was washed with 500μl of 70 % Ethanol (Merk). The DNA pellet was air-dried and resuspended in the 30 μl of deionized water. The DNA was treated with 1.0 μl RNAse (10 mg/ml) to remove any residual RNAs.

**PCR amplification for *transgene* insertion**

PCR amplifications were made to reveal insertion of *cry* gene in CEMB inbred corn line. The gene specific primers (Table 1) were used for amplification. Genomic DNA isolated fromnon-GM corn seeds was used as negative control while for positive control, binary construct containing *cry* genes was used. The PCR reaction mixture contained 100 ng of DNA template, 1X PCR Buffer, 2.5mM MgCl2, 0.2 mM dNTP mix, 1 µM of each forward and reverse primers, 0.5 unit of Taq DNA polymerase (Thermo Scientific) and water was added up to 20 µl. The amplification conditions were; first denaturation at 94oC for 5 minutes, then 35 cycles of denaturation at 95 oC for 30 seconds, annealing at 60 oC for 30 seconds and extension at 72 oC for 1 minute. Final extension at 72 oC for 10 minutes and amplification was performed by ABI 9700 thermocycler.

**Diet Formulation**

For feeding of the subjected birds, four different diets based on corn seeds were prepared that was formulated by mixing specific quantities of non-GM and CEMB-Bt transgenic corn seeds. The diet formulation for control and experimental groups were prepared as described by Hameed et al. (2016) with slight modifications. In the control group, the chicken were fed 50 % non-GM corn and 50 % commercial feed. While the experimental groups, labeled as T50, T40 and T30 contained 50 %, 40 % and 30 % of CEMB-Bt corn respectively in addition to 50 % amount taken from commercial feed (Table 2). Each diet formulation was ground to form porridge.

**Nutritional Analysis of diets**

All the four prepared diet formulations were analyzed for their nutritional contents from Provincial Animal Research Laboratory affiliated with Veterinary Research Institute, Lahore-Pakistan. Three samples from each diet were used for analysis as replicates. The various dietary parameters (dry matter, crude protein, crude fat, crude fiber, ash, phosphorus, Nitrogen-Free Extract (NFE) starch, and soluble sugars) were measured. **Enzyme Linked Immunosorbent Assay (ELISA) to measure Cry toxins (Cry1Ac and Cry2A) in CEMB-Bt corn**

ELISA technique was used to measure the concentration of Cry toxin in all four diets (T30, T40, T50 and control) prepared. The specific Cry toxin concentration in each diet formulation was revealed by using QuantiPlate for Cry1A & Cry2A Kit by Envirologix as per manual. Each sample was used in triplicate and OD was noted at 450 nm through SpectraMax® Plus 384 microplate reader with SoftNax Pro® software. The concentration of specific bound Cry toxin protein was calculated by using the following formula:

 $Concentration of Cry1Ac/2A (µg)=\frac{O.D. of the samples}{Known Concentration \left(Calibrator\right)}\*Calibrator O.D $ **Management of Experimental Animals and feeding trial**

For the Animal bioassay, sixty broiler chicks of one day old were procured by the courtesy of Sabir’s group, Faisalabad Road, Sheikhupura, Punjab, Pakistan. All the chickens were vaccinated against New castle Disease (ND) at the age of 8 days. Later, after 30 days, vaccination against infectious bursal disease was administered to them (Imran et al., 2013). The chickens were kept in a temperature controlled unit according to the protocol described by (Brake and Vlachos, 1998). Further, incandescent lighting was provided for the first seven days of the experiment (Taylor et al., 2003). Chickens were kept at 32oC and then temperature was slowly decreased to 24oC until the completion of the trial. This trial was conducted in the month of February, 2019. Heaters were utilized to maintain the temperature of the chamber. The animals were kept in standard laboratory environments i.e. on floor covered with wooden shavings (Brake and Vlachos, 1998).

For each experimental group that were fed on control, T30, T40 and T50 diet formulations, 15 birds were assigned to one group. The birds were fed for a period of 45 days.

**Feeding trial**

**Total feed and protein consumption**

After completion of feeding period, total feed consumed by each group was calculated. On the basis of feed consumption data we can calculated the total Cry toxin intake by an animal through the following formula.

Total Cry toxin intake by chicken (mg) = $\frac{Total feed consumption \left(kg\right)\*total protein \left(µg\right)}{total number of animals}$

**Sampling Procedure of Animals**

After the completion of feeding assay, blood and organs samples of the animals were collected. Three animals were selected randomly from control and experimental groups for sampling. Blood was collected from the wing vein in Vacutainer (Rossi et al., 2005) for the measurement of serum biochemistry. Total 3 ml blood was drawn from three animals per group and taken into the sterile serum separator tube. Blood samples were centrifuged at 3000 rpm for 10 minutes and serum was collected into the new fresh tubes and stored at 4oC for further analysis. Tissue samples of the vital organs for RNA extraction were preserved in liquid nitrogen in post dissection to preserve and stored at -80oC until use.

**Serum Biochemistry**

Serum biochemistry was performed by the Diagnostic Lab., University of Veterinary and Animals Science, Lahore-Pakistan. The tests include; liver function test (LFTs), AST **(**Aspartate aminotransferase), ALT **(**Alanine Aminotransferase), Liu et al., 2012), ALP (Alkaline Phosphatase) and total protein (TP) along with albumin and globulin. Levels ofcreatinine (CREA), Urea and Uric acid were also investigated for renal function test (RFTs).

**Expression of immune and growth related response genes through quantitative Real-Time PCR**

The relative mRNA expression of selected marker genes for liver (*cGH;* chicken growth hormone gene, *IGF-I* & *II*; Insulin-like Growth Factor), spleen (interleukins; *IL-2* and*IL-β*, *iNOS;* inducible Nitric Oxide Synthase, toll-like receptors; (*TLR-05* and *TLR-15*) and intestine (*mucin* gene) was measured. For normalization, *28S rRNA* was used as reference gene (Bhanja et al., 2014). The assays were performed in PikoReal PCR (Thermo Scientific). Primers sequence and details are depicted in the table 1.

**RNA extraction and cDNA Synthesis**

For total RNA extraction from liver, spleen and intestine samples taken from the sacrificed chicken, optimized protocol described by Toni et al. (2018) was adopted. 100 mg of tissue sample was taken from cryogenic vial and ground in liquid nitrogen for RNA isolation. Concentration of total RNA was measured by using NanoDrop (ND1000) by Biocampare. In order to synthesize cDNA, kit was obtained (Thermo Scientific’s; Catalog #K1622) and following the protocols described in the Kit manual.

**qPCR for the genes expression**

In order to quantify and compare the expression of *mucin,* growth related and and immune response from intestine, Liver and spleen through qPCR. For this purpose, SYBR Green dye kit (Themo Scientific; cat# K0229) and PikoReal Real-Time PCR System (Thermo Scientific) was utilized to perform the Real-Time PCR. Reaction mixture of each cDNA sample was prepared in triplicates. For the preparation of the reaction mixture 1μl of each primers was added, 5.5μl Maxima SYBR Green master mix (2X), 50 ng of cDNA and 1.5 μl water (nuclease-free) up to 10μl. After dispensing all the components of master mix into PCR tube, master mix was mixed properly and then aliquoted into individual wells of Real-Time PCR plate. *28s* gene was used as internal control reference gene. After covering 96 wells plate with septa, plate was centrifuged briefly for thorough mixing of the reaction components. Plate was kept on ice and dark throughout the reaction preparation. qPCR reaction conditions were denaturation at 95oC for 3 minutes, 35 cycles of denaturation at 95oC for 30 seconds, annealing at 59oC for 30 seconds and extension at 72oC for 30 seconds. The Cq values of the samples were analyzed by PikoReal™ Software 2.1 (Thermo Scientific). The gene expression data was analyzed using Livak method (Livak et al., 2013).

**Statistical Analysis**

The body weight and daily feed consumption by the feeding chicken was graphically represented by using the average values. Compositional diet analysis, various biochemical tests, and gene expression data were subjected to statistical analysis and Microsoft Excel was used to calculate mean and standard deviation. Then performed statistical analyses (1-way ANOVA) by using software Graph Pad Prism (version 5.00.288) for Windows 10. Post-test applied was Dunnet’s test if p>0.05 then values are non-significant and vice versa.

**RESULTS**

**Transgene (*cry1Ac/cry2A*) insertion and protein concentration in CEMB-Bt Corn seeds**

The transgene *cry1Ac/cry2A* were amplified from the CEMB-Bt corn samples to confirm their insertion. It was found that a specific fragment of 769bp was amplified in GM samples while no such amplification was observed in samples taken from the non-transgenic corn seeds (Figure 2). While the concentration of Cry2A + Cry1Ac proteins in CEMB-Bt corn seeds in four diet formulations were 1.62 µg/gm, 1.32 µg/gm, 0.96 µg/gm and 0 µg/gm in T50, T40, T30 and control group respectively (table 3).

**Nutritional Analyses of diets**

The percentage of the dry matter percentage, crude protein, crude fat, NFE starch+ soluble sugar and phosphorus measured. Results has confirmed that the nutritional composition of each of the four diet formulations (control, T30, T40, T50) didn’t exhibit any significant differences in dietary components among control and experimental groups (table 4).

**Total feed and Cry protein consumption**

During the 45 day feeding trial, no any noticeable difference was observed in feed consumed by subjected chicken in all four groups. Specifically, the control group animals consumed about 57.85 kg of feed while the average feed consumed by T50, T40 and T30 group was 56.36 kg, 58.36 kg and 56.95 kg respectively. Based on the average Cry protein concentration in diet fed to bird, the estimated Bt protein taken by an animal was 0 mg, 6.09 mg, 5.14 mg and 3.64 mg by control, T50, T40 and T30 group respectively (Table 5).

**Serum Biochemistry**

Serum biochemistry analyses includes LFTs (Liver function tests) and RFTs (Renal Function tests). For liver function test, no statistical difference between the values of enzymes expressed by liver; ALT, AST and ALP was found (*p*>0.05) (Table 6). Results of serum protein analysis parameters i.e. total protein, Albumin, and Globulin were also in the normal range and showed non-significant difference among the values of the control and transgenic groups (*p*>0.05) as shown in table 6. In renal function test, non-significant difference was observed between the values of Creatinine (CR) and Uric acid (UA) in different groups (*p*>0.05) as shown in table 7. Results of renal function test among groups were comparable.

**Expression of mucin, growth-related and immune response genes**

The gene expression data of mucin, growth-related and immune response genes were analyzed using 2^(-ΔΔCt) Livak method. No significant difference was found in the relative gene expression among control and experimental groups (p>0.05) as shown in the figures (figure 3.1-3.3) except *GH* gene expression of control group is slightly low values and significant different to the transgenic groups (p<0.05)



**Figure 1:** Construct map transformed in to the CEMB Bt corn variety showing *Cry1Ac* and *Cry2A* genes.



**Figure 2:** Transgene (*Bt*) detection in GM and non GM corn. L; 1kb Ladder, 1; Non-GM Corn and 2; GM Corn, 3; positive Control



**Figure 3.1:** Relative expression of Mucin gene related to intestinal tract development of control and transgenic corn fed groups chicken (p>0.05; n=3).



**Figure 3.2:** Relative expression of chicken growth related gene (*cGH*; growth hormone *IGF*; Insuline like Growth Factor I and II). *GH* expression of control group is slightly low values and significant different to the transgenic groups (p<0.05; n=3). Expression of *IGF-I* and *II* gene shown no significant difference among control and transgenic groups (p<0.05; n=3).



**Figure 3.3:** Relative expression of chicken immune related gene. Expression *of IL-2, IL- β, TLR-04, TLR-15* and *iNOS* genes showed non-significant difference comparing to control and transgenic group (p>0.05; n=3).

**Table 1:** List of the primers for bt genes and Quantitative Real-Time PCR Primers

|  |  |  |  |
| --- | --- | --- | --- |
| Primers name  |  Primer Sequence 5' to 3'  | Annealing temperature (oC) | Product size (bp) |
| **Bt gene** |
| *Bt-F* | ATCTTCACCTCAGCGTGCTT | 62 | 769bp |
| *Bt-R* | GGTGGCACATTGTTGTTCTG |
| **Major Growth Related Gene** |
| *IGF-I*-F | GGTGCTGAGCTGGTTGATGC | 58 | 203 |
| *IGF-I*-R | CGTACAGAGCGTGCAGATTTAGGT |
| *IGF-II*-F | GGCGGCAGGCACCATCA | 58 | 215 |
| *IGF-II*-R | CCCGGCAGCAAAAAGTTCAAG |
| *cGH*-F | CACCACAGCTAGAGACCCACATC | 58 | 201 |
| *cGH*-R | CCCACCGGCTCAAACTGC |
| ***Mucin* gene** |
| *Muc*-F | CTGGCTCCTTGTGGCTCCTC | 58 | 242 |
| *Muc*-F | AGCTGCATGACTGGAGACAACTG |
| **Immune Response Gene** |
| *IL-2*-F | CCCGTGGCTAACTAATCTGCTG  | 57 | 287 |
| *IL-2*-R | TGAGACACCAGTGGGAAACAGT |
| *TLR-04*-F | GTTCCTGCTGAAATCCCAAACACC | 58 | 239 |
| *TLR-04*-R | GCCAAGAGCCACGAGACTCCAAA |
| *TLR-15*-F | GTGAGAATGGGCTGGTACTGGTG | 58 | 203 |
| *TLR-15*-R | CCAAGTACAGGATGCCCTGGT |
| *IL-**1β* –F | CATGTCGTGTGTGATGAGCGG | 57 | 208 |
| *IL-**1β* –R | GCTGTCCAGGCGGTAGAAGATGAA |
| *iNOS*-F | GTGTTGTGTGCTTCCACTGC | 59 | 215 |
| *iNOS*-R | AACACCTCCAAAGCCCTAGC |
| **Reference Gene** |
| *28s*-F | CAGGTGCAGATCTTGGTGGTAGTA | 58 | 273 |
| *28s*-R | GCTCCCGCTGGCTTCTCC |

Note: Bt (*Bacillus Thuringiensis*; *cry1Ac* and *cry2A* genes) IGF (Insulin-like Growth factor), GH (chicken Growth Hormone), Muc (mucin), IL (interleukin), iNOS (inducible Nitric Oxide Synthase), TLR (Toll-like receptor) and 28S = 28S rRNA.

**Table 2:** Experimental diets GM corn compositions for control and transgenic groups.

|  |  |  |  |
| --- | --- | --- | --- |
| **Diet groups** | **Commercial Diet** | **CEMB-Bt corn seeds** | **Non-GM Corn****corn seeds** |
| **Control** | 50% | 0% | 50% |
| **T50** | 50% | 50% | 0% |
| **T40** | 50% | 40% | 10% |
| **T30** | 50% | 30% | 20% |

Note: Commercial diet for broiler chicken purchased from local market.

**Table 3:** Quantification of Cry1Ac and Cry2A protein in the GM corn seeds and experimental diets.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Transgenic corn (%)** | **Cry1Ac** | **Cry2A** | **Cry1Ac+Cry2A** |
| GM corn Seeds | 100% | 1.4 µg/gm of seeds | 1.82 µg/gm of seeds | 2.22 µg/gm of seeds |
| Control (diet) | 0% | 0 µg/gm of diet | 0 µg/gm of diet | 0 µg/gm of diet |
| T50 (diet) | 50% | 0.70 µg/gm of diet | 0.92 µg/gm of diet | 1.62 µg/gm of diet |
| T40(diet) | 40% | 0.58 µg/gm of diet | 0.74 µg/gm of diet | 1.32 µg/gm of diet |
| T30 (diet) | 30% | 0.42 µg/gm of diet | 0.54 µg/gm of diet | 0.96 µg/gm of diet |

**Table 4:** Nutritional Analyses of diet for each group

|  |  |  |
| --- | --- | --- |
| **Components (%)** | **Treatments** |  |
| **Control** | **T50** | **T40** | **T30** | **p-value** |
| Dry Matter | 91.52±1.24 | 93.02±3.04 | 91.85±4.38 | 91.94±4.49 | 0.9571 |
| Ash | 3.84±0.96 | 3.97±1.19 | 3.81±1.13 | 3.84±0.37 | 0.9967 |
| Crude Protein | 12.20±1.37 | 13.40±0.71 | 12.80±1.67 | 12.83±1.37 | 0.7516 |
| Crude Fat | 1.74±0.23 | 1.63±0.34 | 1.67±0.25 | 1.68±0.26 | 0.9716 |
| Crude Fiber | 7.48±0.83 | 7.37±0.95 | 7.40±1.06 | 7.37±0.95 | 0.9987 |
| NFE (%) Starch + Soluble Sugars | 64.60±1.73 | 64.34±4.64 | 63.83±4.10 | 63.62±4.23 | 0.9882 |
| Phosphorus | 0.16±0.08 | 0.15±0.06 | 0.16±0.05 | 0.15±0.07 | 0.9955 |

Values in the table are the mean of the three replicates. Control, T50, T40 and T30 containing 0%, 50%, 40% and 30% GM corn respectively along 50% with commercial chicken feed. All nutritional values of control diet are non-significant with transgenic diet (p>0.05).

**Table 5:** Approximate intake of Bt protein by per animal of the groups.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **A: (total protein) (µg/gm of diet)** | **B: Total feed consumption of Feed (kg)** | **Total protein intake by per animal animals =**$$\frac{A X B}{15 (no. of animals in each group }$$ |
| Control | 0 | 57.85 | 0 mg |
| T50 | 1.62 | 56.36 | 6.09 mg |
| T40 | 1.32 | 58.36 | 5.14 mg |
| T30 | 0.96 | 56.95 | 3.64 mg |

**Table 6:** Liver function parameters of control and transgenic groups

|  |  |  |
| --- | --- | --- |
| **Liver function parameters** | **Treatments** | **p-Values** |
| **Control** | **T50** | **T40** | **T30** |
| AST (U/L) | 290.00±58.85 | 294.00±61.61 | 280.33±29.19 | 281.33±57.01 | 0.9855 |
| ALT (U/L) | 21.00±2.00 | 21.00±3.46 | 21.00±1.00 | 20.67±2.08 | 0.9972 |
| ALP (U/L)  | 2714.6±206.3 | 2715.3±95.0 | 2603.7±183.9 | 2757.3±236.7 | 0.7787 |
| Total Protein (g/dL) | 2.97±0.31 | 2.97±0.15 | 3.00±0.36 | 3.04±0.25 | 0.9875 |
| Albumin (g/dL) | 1.63±0.15 | 1.77±0.22 | 1.70±0.40 | 1.67±0.21 | 0.9237 |
| Globulin g/dL | 1.37±0.15 | 1.43±0.23 | 1.50±0.26 | 1.57±0.21 | 0.7048 |

Note: Liver function parameters (ALT, AST and ALP, total protein, Albumin, and Globulin) values were non-significant of control and transgenic groups animals (p>0.05; n=3).

**Table 7:** Renal function parameters of control and transgenic groups

|  |  |  |
| --- | --- | --- |
| **Renal function parameters** | **Treatments** | **p-Values** |
| **Control** | **T50** | **T40** | **T30** |
| UR (mg/dL) | 1.73±0.25 | 1.60±0.300 | 1.47±0.15 | 1.63±0.32 | 0.6944 |
| CR (mg/dL) | 0.23±0.06 | 0.20±0.10 | 0.17±0.06 | 0.20±0.10 | 0.8490 |
| UA (mg/dL) | 5.40±1.08 | 5.27±0.74 | 5.17±1.31 | 5.33±0.75 | 0.9930 |

Note: Renal function test of control and transgenic groups animals. Values of UR; Urea, CR: Cretinine and UA; Uric Acid (UA) showing no significant difference among control and transgenic groups. (p>0.05; n=3).

**Discussion**

Agriculture has revolutionized by the transgenic crops in the recent years, however adaptation at commercial level require minimized biosafety concerns. In this context, several toxicological assessments related to transgenic crops have previously been conducted across the globe that revealed little information about their validity (Ibrahim and Okasha, 2016). Thorough assessment of GM crops on animal models along with toxicity assessments is mandatory (Alexander et al., 2007; Trabalza-Marinucci et al., 2008). In the present study, 45 day feeding trial of locally developed insect resistant CEMB-Bt corn on broiler chickens and any potential effect of transgenes on growth, development, immunity and serum chemistry of subject bird was reported.

In this study, a defined concentration of the insecticidal Cry toxins (Cry1Ac and Cry2A) were fed to broiler chicken among four different groups. Concentration of the transgenic protein was estimated through ELISA which is a common technique used for the quantification of the transgenic proteins in various crops (Bashir et al., 2005; Zhang et al., 2016).

 Rigorous safety evaluation of a GM crop is warranted if the nutritional attributes has been significantly altered in transgenic line. Hence, to overcome this, measured the nutritional composition of each diet prepared and found no significant difference. Castillo et al. (2004) compared nutritional compositions of the diets with their considerable equivalences in a similar study. It has been reported from the previous study that *Bacillus thuringenesis* derived insecticidal gene (*Cry1Ac*) expressing insect resistance proteins could not change the dietary compositions of cotton (Tripathi et al., 2011). Another studies also reported that the insertion of foreign DNA into plants did not alter the nutritional values of their seeds (Chrenková et al., 2002; Salisu et al., 2018).

It has been documented that chronic feeding of the GM feed might have more authentic outcome as compared to short term trials. This study reported a 45 day feeding trial which is comparable with others feeding bioassays conducted by (Řehout et al. (2009) on mixed population of broiler chickens fed Bt corn for 42 days. Another study performed in Poland on broiler chickens of mixed population fed Bt corn or RR soybean for 42 days (Reichert et al., 2012). Similarly, bioassay trail was performed in Pakistan with mixed population of broiler chickens through feeding of Bt sugarcane with commercial diet for 120 days (Hameed et al., 2016). Consumption of feed, weight gain and growth rate of chickens of all the experimental and control groups were analyzed, no changes were recorded in all these parameters. The results of this study reported that feeding transgenic corn even up to 50% level of composition do not significantly effects the growth of the broiler chickens. Similar finding were reported by Czerwiński et al. (2015) while feeding assay on broiler chicken fed with transgenic corn (c*ry1Ab*) did not differ from those fed conventional corn. Similarly the inclusion of transgenic corn in broiler chickens feed did not change the nutritional compositions and their performances (Anderson et al., 2019). Another comparable results were recorded, when Bt cotton was given to catfish and Northern Bobwhite (Quail) as feed (Hamilton et al., 2004). Thus, bird performances were not significantly influenced by the poultry feed containing transgenes reported by Chesson and Flachowsky (2003).

In serum biochemistry analysis, concentration of different enzymes is used to analyze the liver and kidney functions (Harper, 1971). Most of the serum proteins are produced in the liver and perform various tasks like maintenance of blood volume, hormonal transportation, metabolic regulation and providing protection against foreign invaders (Rezende et al., 2017). In current study, serum biochemistry (LFTs, RFTs and lipid profile) parameters were analyses that shown the non-significant differences when compared to the values of control. Řehout et al. (2009) conducted a feeding assay of Bt corn and found that the liver enzyme and total protein content of boiler chicken fed Bt corn (MON810) were found no significant difference and within physiological range. In 120 days feeding trial, broiler chickens fed GM sugarcane (c*ry1Ac*) along with commercial diet found non-significant difference in the ALT, AST, ALP, creatinine, and urea (Hameed et al., 2016). Similarly, another study, non-significant difference in the values of ALT, AST, urea, bilirubin, creatinine were reported in layer chickens fed Bt cotton *CEMB-cry1Ac+cry2A* genes (Imran et al., 2013). In 49 days feeding trial, GM corn (Cry1Ab and EPSPS proteins) were not harmfully affect the serum biochemistry and hematology of Japanese quails (Zhang et al., 2021).

 The pathological process caused by any toxicant regulates the expression of number of genes including immunity associated genes. Analysis of the level of relative expression of immunity and developmental related genes can be assessed by qPCR (Laptev et al., 2019). The relative gene expression of immune related gene upon feeding of GM crops is poorly understood. In the current study, feeding Bt corn did not cause any observable effects on splenic relative expression of selected genes *IL-1β, IL-2, iNOS, TLR4*, and *TLR15* of the broiler chicken up to 50% along with commercial diet.

Growth hormone (GH) controls growth through insulin-like growth factor-I (IGF-I). IGF-I is produced in liver under the influence of GH (Nguyen and Anh, 2015). Insulin-like growth factor-I and II control the growth of different cells like fibroblasts, preadipocytes, and chondrocytes by increasing DNA synthesis, glucose consumption, and tissue development. IGF-II increases cellular development, differentiation, and viability (Yan et al., 2017). Our findings were relative GH gene expression is low and significant different (p<0.5) while non-significant difference in relative *IGF-I* and *IGF-II* genes expression among control and from the transgenic groups was observed. Nutrient digestion and absorption is influenced by the Mucin which is major constituent of the mucus layer. Dietary components have the potential to induce changes in mucin dynamics. Higher expression of mucin gene triggered the number of goblet cells and production of acidic mucin in the GIT of a chick (Bhanja et al., 2014). No significant difference was found in the relative gene expression of mucin gene among control and transgenic groups.

**Conclusion**

In Conclusion, the results obtained from the feeding of GM corn seeds containing two *Bt* genes *(cry1Ac+cry2A)* to growing broiler chickens have demonstrated no severe health concerns on their growth and performance. Exposure to GM corn did not induce significant alterations in the gene expression or in the specific growth related and immune responses in the chicken and no harmful effects were detected on monitoring the changes in specific biochemical parameters. Thus the overall results of this study suggesting that CEMB Bt corn expressing the Cry1Ac and Cry2A protein is safe to use as a feed in poultry industry and providing consumers the safety assurance of the transgenic feed ingredients.

**Competing interests**

No potential conflict of interest relevant to this article does exist.

**Author’s Contributions**

**Zakiya Javed:** Investigation. **Fazeel Laraib:** Investigation. **Asmatullah:** Supervision. **Bushra Tabassum:** Validation. **Abdul Munim Farooq:** Resources. **Anwar Khan:** Review and editing. **Muhammad Tariq:** Data curation, original draft preparation. **Idrees Ahmad Nasir:** Supervision and Conceptualization.

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