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BIOSAFETY ASSESSMENT OF LOCALLY DEVELOPED TRANSGENIC SUGARCANE

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ABSTRACT

Genetically modified organisms have become obligatory to adopt certain precautions to overcome the risks related to release of GMOs. Through this study, an attempt was made to evaluate bio-safety concerns of transgenic sugarcane with insect resistant gene, Cry1Ac. In short term feeding trial of 120 days, 20 broiler chicks divided into four random groups were caged separately. Group-I fed on commercial chicken diet; group-II fed on non-transgenic sugarcane diet; group-III fed on transgenic diet (commercial to transgenic ratio was 1:1) while group-IV was fed on high dose transgenic diet (commercial to transgenic ratio was 1:2). Body weights of chicks increased normally in all four groups during the course of study. Biochemical tests and molecular tests done on chick's blood reported no significant difference among assorted groups. At the termination of the experiment, chicks were slaughtered and dissected vital organs were used for histological studies. It was found that tissues belong to group 3 and 4 were morphologically and histological similar to group 1 and 2. Also, no mRNA expression was observed in total RNA isolated from chicken's tissues. These findings suggest that there was no deleterious or harmful effect of the GM sugarcane on the chick's health and no transgene fragment has transferred from GM crop to animal's blood neither in their vital tissues. Conclusively, it is suggested that at least in short-term period feeding trial, the genetically modified sugarcane doesn't not have adverse effect on animals feeding them.

Key words: Bt Sugarcane, Feeding Trial, Broiler chicks, Biosafety assessment.

INTRODUCTION

Today, a major challenge for mankind is how to increase world access to adequate food without depleting nonrenewable natural resources and causing environmental damage through opting nature friendly approaches (Hallerman and Grabau,2016; Mobeen and Latif, 2016).Rapid increase in the world population and the resultant demand for food has forced the experts to evolve and adopt new strategies to overcome hunger, malnutrition, insect/pest attack, herbicides use, salinity and tolerance of crops to adverse conditions. The developed world has multiplied its crop yield through mechanized farming and by utilizing hybrid technologies but these technologies have yet to gain ground in Pakistan. Genetic engineering includes, improving crop production and introducing new traits, such as enhanced nutrients, temperature resistance, or the ability to grow in saline soils etc (Ali *et al*., 2016).

Sugarcane crop is one of the most important crops worldwide as it contributes approximately 70% of the total sugar yield. Despite expansion in production over years, increase in productivity per unit area has been very low in Pakistan. The average sugarcane production in the country remained static between 45-50 tons/ha. A number of biotic and abiotic factors are limiting yield of sugarcane. Borers are also the major pests of cane crop.

Currently the pest is controlled by application of granulated pesticide in the plant whorl manually. Synthetic insecticides cause the contamination of water and food sources and killing of non-target insects while their continuous use develops resistance in pests. *Bacillus thurigiensis (Bt)* is a gram positive bacterium and can be used as a promising bio-pesticide (Tang *et al*., 2012). Only few insecticidal sprays are required which save time, money, health risks and environmental hazards. Bt sugarcane is successfully being cultivated in Australia, Brazil and several other countries of the world. Transgenic plants expressing insecticidal proteins from the *Bacillus thuringiensis (Bt)* were first commercialized in 1996 with the consent of scientists, regulators and environmentalists (Bates *et al.*, 2005).

Bt sugarcane developed by CEMB has been genetically modified which is resistant to attack by lepidopteran insect pests, as it expresses the Cry1Ac protein. The codon was optimized for the isolated Cry1Ac gene (Khan *et al*., 1995) according to the sugarcane genome for maximum expression. Although the bacterial Cry1Ac protein has been extensively used as an organic insecticide (Betz *et al*, 2000), however its expression in transgenic sugarcane could potentially alter

its structure which may render it allergenic or otherwise harmful upon ingestion(Prescott *et al*, 2006) as the sugarcane is used as a component of feed for cattle.Hence, the application of geneticmodification technology and the usage of genetically modified products must be accompanied byrisk assessment of their potential impact on food, feed and environment safety(Kumar *et al.*, 2008).

Uptill now, several studies have been conducted around the world to detect any potential effect of Bt crops on non-target insects and animals through vertical or horizontal gene flow(Pinto *et al.*, 2013; Wang *et al.*, 2013).One of the most challenging problems around the world is risk assessment and bio-safety study of genetically modified crops. Several biosafety studies have suggested that genetically modified plants are safe and have the same nutrition value as their conventional counterparts (Aumaitre *et al*., 2002; Aeshbacher *et al.*, 2005; Bertoni and Marsan, 2005; Calsamiglia *et al*., 2007; Domingo *et al.*, 2011; Liu *et al.*, 2012;Mezzomo*et al.*, 2013). Duggan *et al*. (2003) conducted a short term biosafety trial of five hours. The sheep were fed on GM insect-resistant maize grains followed by amplification of transgene through PCR assay.

The focal point of the current study was the assessment of Bt sugarcane modified by CEMB, in biosafety perspective.

MATERIALS AND METHODS

GM transgenic sugarcane: The Bt sugarcane plants modified with Cry1Ac gene were kindly obtained from Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore. Cry1Ac gene was isolated from local *Bacillusthuringiensis* strain by Khan *et al*. (1995). Further, the codon of the gene was optimized according to sugarcane genome by utilizing various bioinformatics tools and transformed in sugarcane to develop CEMB-Bt sugarcane. Transgene was expressed under the control of ubiquitin promoter. Transgenic plants were confirmed for the presence of transgene through PCR and Dipstick assay. For amplification by PCR, Cry1Ac gene specific primers forward 5’ GTTCTGCCCAAGGTATCGAA 3’ and reverse 5’ GGCACATTGTTGTTCTGTGG 3’were designed by using primer3 software version 3 (primer3plus.com/web_3.0.0/primer3web_input.htm).
PCR was performed in thermal cycler ABI, 9700. The reaction mixture contained 1X PCR reaction buffer, 0.1mM of dNTPs, 1pmole each of forward and reverse primer, 1 unit of Taq DNA polymerase (Fermentas) and 50ng of template DNA was used. PCR reaction was carried out in a thermal cycler (GeneAmp PCR system 9700, ABI) with the following thermal cycling parameters: initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 40 seconds, 54°C for 40 seconds and 72°C for 40 seconds and a final extension of 10 min at 72°C. Amplified products were resolved on 0.8 % agarose gel.

Further, transgenic sugarcaneplants were further confirmed by dipstick assay where Cry1Ac gene-specific monoclonal IgG coated sticks (Envirologix, Brazil). The assay was done by simply dipping the coated stick in crude plant protein extract dissolved in buffer for 15-30 minutes at room temperature. Appearance of the test band on dipstick will be observed subsequently.

Selection of Chickens: Forty broiler 3-day old chickens were selected for ashort term (120-days) GM feeding trial. Chickens were fed on normal diet for two weeks in optimized conditions and subsequently transferred to group specific diet. Chickens were randomly divided into four groups with ten chicks in each group. Each group was fed on commercial chick feed in combination with GM sugarcane feed, in four different ratios.Groups were labeled according to their feed combinations as group-I refer to control which fed totally on commercial diet;group-II refers to non-transgenicgroup where chicks were fed on GM sugarcane diet mixed with commercial diet in 1:1 ratio;group-III denote the transgenic low dose group who fed on GM sugarcane diet mixed with commercial diet in 1:3 ratiowhile group-IVdenote high dose transgenic group whose diet include GM sugarcane mixed with commercial diet in 3:1 ratio.The weight of chickens was recorded after every 15days for a total study period of 120days.

Blood Sampling and Biochemical Testing: To check the presence of Cry1Ac protein in GM feeding animals, blood samples were collected. These samples were preceded for any transgene presence detection through PCR and biochemical tests. For PCR, Cry1Ac gene specific primers were used while as internal control, beta actinprimers; forward 5’ CCACAATGTACCCTGGCATT 3’ and reverse 5’ CAGACAGAGT ACTTGCGCTC 3’ were used to amplify the transgene.

Following biochemical tests; Blood urine nitrogen, Alanine transferase, Aspartate transferase, Creatinine, Lactate dehydrogenase and Cholesterol were performed. These tests will define the functioning of vital organs and physiology of the animals.

Histological analysis of vital organs: At the termination of feeding trial, animals were sacrificed and their vital organs; heart, liver and intestine were removed for further morphological and histological studies. Morphological variations in terms of organ structure, weight and maturity were observed. Simultaneously, collagen deposition and fibrosis in the heart, liver and intestine was marked. Sectioning was done with microtome followed by staining with hematoxyline and eosine.

These sections were observed under microscope for comparison.

RESULTS

GM transgenic sugarcane plants: PCR and dipstick assay of the genetically modified sugarcane confirmed the presence of the transgene Cry1Ac. A 418bp gene fragment was amplified from GM sugarcane leaves as depicted in figure 1A while figure 1B confirmed the presence of Cry1Ac protein in transgenic sugarcane as revealed through dipstick assay.

Diet analysis: Feeding diet analysis was done by PCSIR, Lahore which confirmed that the total energy and nutrient content was almost same among all experimental groups (table 1).The total energy of the feed in control group was 353kcal/100gm while in group-III and group-IV the total energy of the feed was 373 and 328kcal/100gm respectively. Similarly, the %age of the protein content of the diet was 12.32, 12.15 and 11.94 in group-I (control), group-III (low transgenic diet) and group-IV (high transgenic diet) respectively.

Biochemical Analysis of Blood: The toxicological effect of the genetically modified sugarcane diet on subjected chicken was evaluated by the biochemical analysis of the blood. It was found thet no statistically significant difference in the values of alkaline phosphatase, alanine aminotransferase, asparatate aminotransferase, cholesterol, uric acid, eosinophil, hemoglobin, lactate dehydrogenase, lymphocyte, packed cell volume, red blood cells, total lymphocyte count, urea and creatinine was found. The results values lie within in the normal range(Figure 2).

Transgene Expression profiling in experimental animals post feeding trial: DNA extracted from blood samples of animals in assorted groups were used to amplify transgene, if any, through PCR assays.The results clearly demonstrated that *traasgene (CrylAc) was* absent in the samples as no fragment was amplified in test samples as compared to the positive control,however amplification was evident for beta actin gene which was used as an internal control. Similarly, no amplification of transgene was obtained in RNA extracted from heart, liver and intestine tissues (Figure 3).

Morphology and Histological Comparison of vital organs among assorted groups: With respect to weight of chickens, no significant difference was found as presented in linear regression graph**(**figure 4). R^2 shows the correlation coefficient. R value gives the proportion of the variance of one variable that is predictable from the other variable. Similarly, no significant difference was found in weight of vital organs among four assorted groups (figure5).

Histologically, there was no significant difference in color, texture, weight, organ serosa's, appearance of spots etc. and in the general appearance of vital organs of experimental groups as depicted in figure 6, 7 and 8. The experimental groups II, III and IV share same cell morphology, texture as of control group (group-I). Conclusively, there was no significantdifference in the cellular architecture of the subjected organs in group I, group II, group III and group IV (Figure6, 7&8).

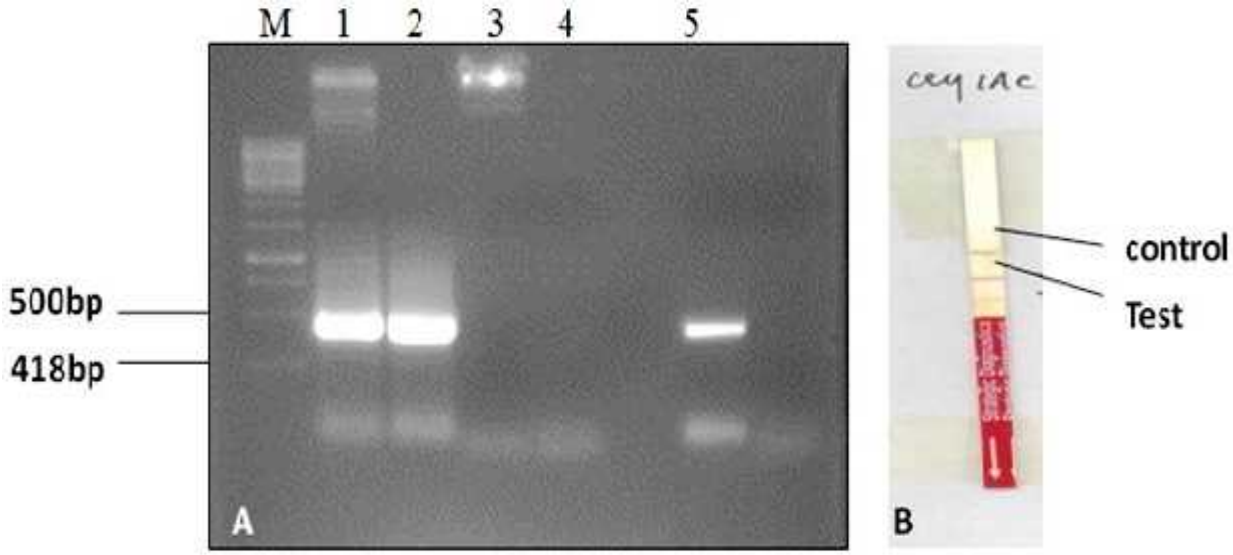

Figure 1. Confirmation of transgenic sugarcane plants for Cry1Ac gene through A): PCR assay and B): Dipstick analysis of transgenic plants. Where M: 1kb DNA ladder; lane 1: positive control, Lane 2: sample # 1, lane 3: sample # 2, lane 4: sample # 4, lane 5: negative control.

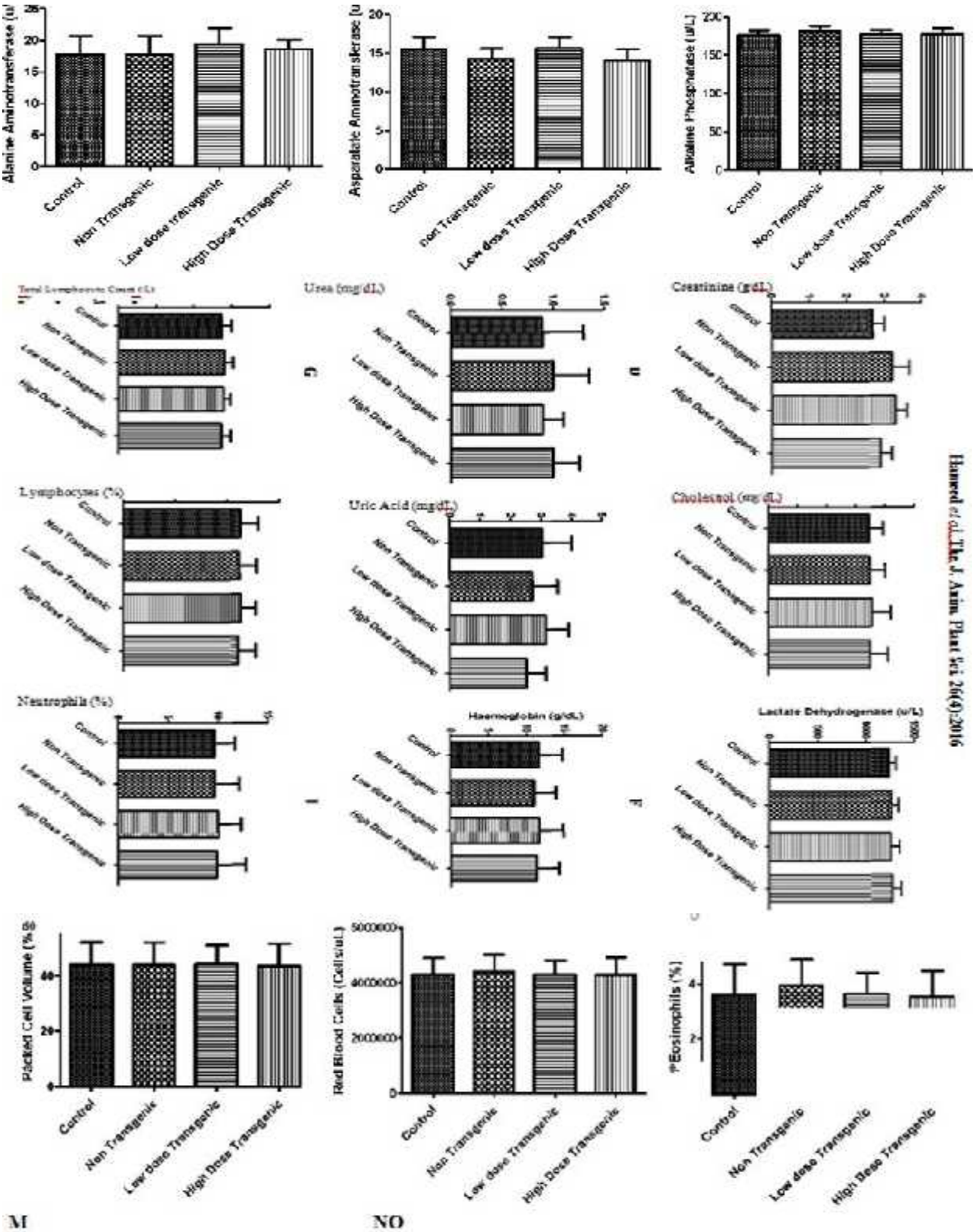

Figure 2. The biochemical tests of chick's blood from four assorted groups. (A) Alanine Aminotransferase (B) Asparatate Aminotransferase (C) Alkaline Phosphatase (D) Creatinine (E) Cholestrol(F) Lactate Dehydrogenase (G) Urea (H) Uric Acid (I)Heamoglobin (J) Total Lymphocyte Count (K) Lymphocytes (L) Neutrophil (M)Packed Cell Volume (N)Red Blood Cells (O) Eosinophils.

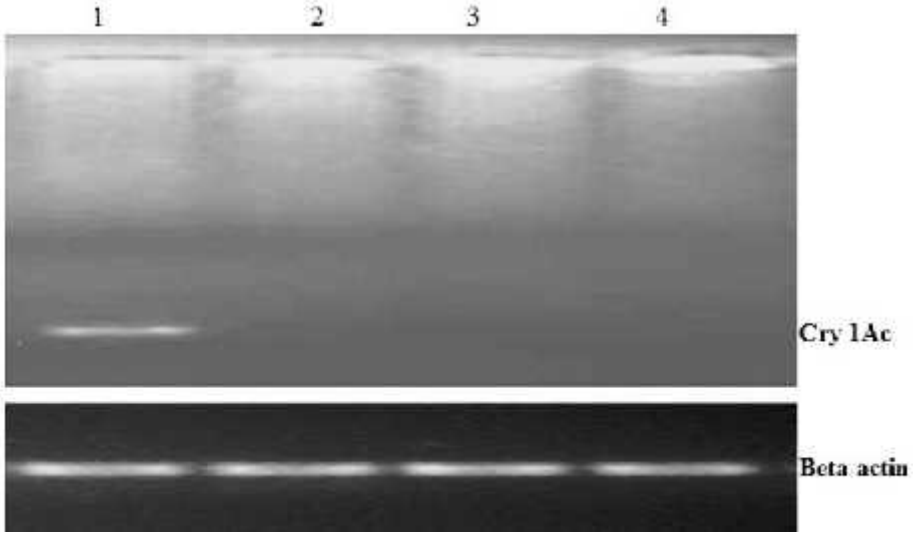

Figure 3. PCR amplification of Cry1Ac transgene in chick's blood samples collected from vital organs of four experimmental groups. While Beta actin gene amplification was used as reference gene. Lane 1: positive control; Lane 2: heart sample; Lane 3: intestine sample; Lane 4: Liver sample.

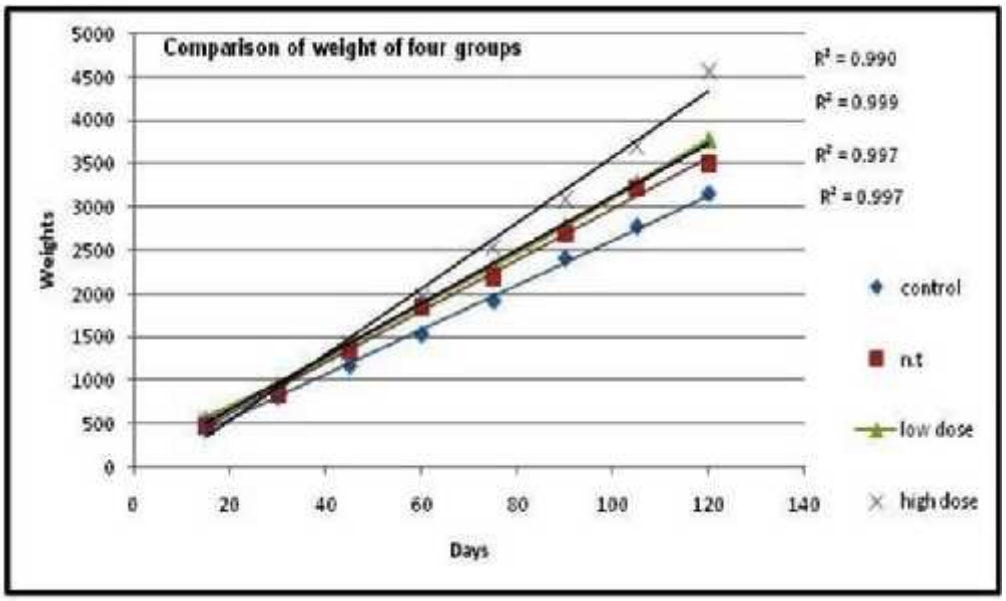

Figure 4. The line graph plotted between the weight and days shows gradual increase in the weight of four groups.

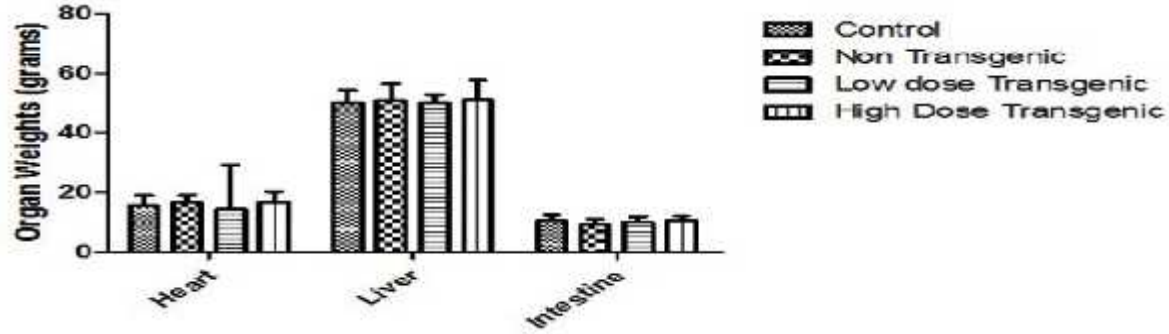

Figure 5. Weight of organs for randomly selected chickens from four groups, control, non-transgenic, low dose and high dose.

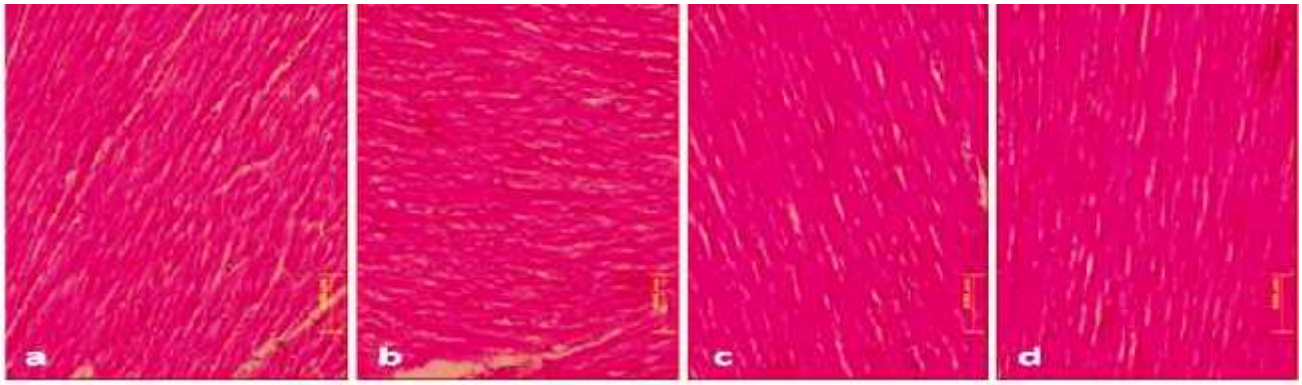

Figure 6. Comparison of cellular architecture of heart between randomly assorted groups (a): The cellular structure of cardiac tissues of the chickens in control group fed with commercial diet in group-I, (b) indicates the cellular structure of cardiac tissues of the chickens fed with non- transgenic sugarcane, group-II, (c) indicates the cellular structure of cardiac tissues of the chickens fed the commercial diet + low dose of transgenic sugarcane, group-III, (d) indicates the cellular structure of cardiac tissues of the chickens fed on commercial diet + high dose of sugarcane, group-IV.

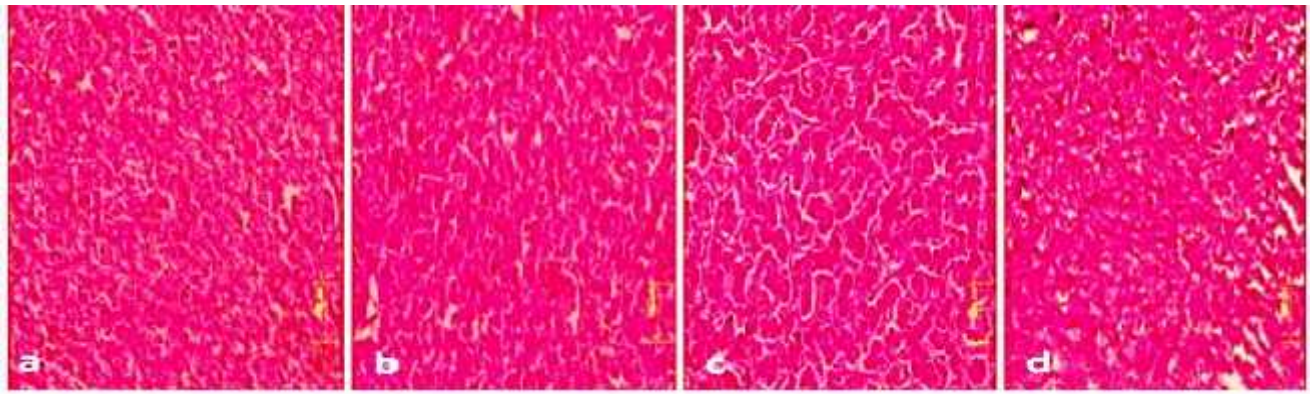

Figure 7. Comparison of cellular architecture of liver between control, non- transgenic and experimental groups (a): The cellular structure of liver tissues of the group-I (control) chickens, (b) the cellular structure of liver tissues of the chickens in group-II , (c) indicates the cellular structure of liver tissues of the chickens in group-III, and (d) indicates the cellular structure of liver tissues of the chickens in group-IV.

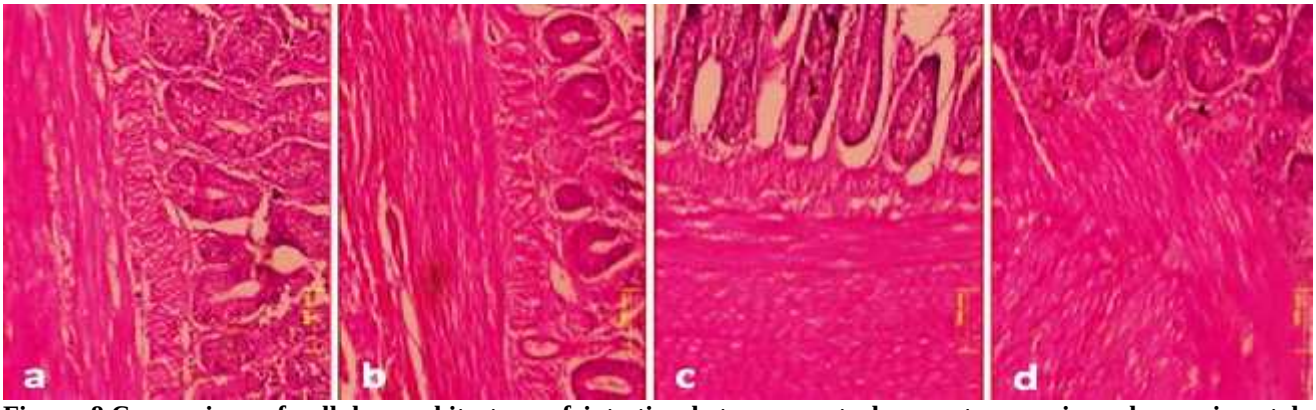

Figure 8.Comparison of cellular architecture of intestine between control, non- transgenic and experimental groups (a): The cellular structure of intestine tissues of the chickens fed with commercial diet in group-I (control), (b) indicates the cellular structure of intestine tissues of the chickens fed with non- transgenic sugarcane in group-II, (c) indicates the cellular structure of intestine tissues of the chickens fed the commercial diet + low dose of transgenic sugarcane in group-III and (d) indicates the cellular structure of intestine tissues of the chickens fed on commercial diet + high dose of sugarcane in group-IV.

Table 1. Nutritional evaluation of the diet samples fed to four assorted groups of chicken. The %age values were determined by PCSIR Laboratories, Lahore - Pakistan.

	Group 1	Group 3	Group 4
Moisture (%)	7.29	5.37	5.62
Ash (%)	10.07	9.62	8.95
Protein (%)	12.52	12.15	11.94
Fat (%)	5.57	12.52	6.30
Fibre	5.85	7.20	11.20
Nitrogen free extract (NFE) (%)	58.7	53.14	55.99
Energy (Kcal/100gm)	335	373	328

DISCUSSION

The recent developments in biological science and technology have brought the world into a new era of biotechnology (Hatti-Kaul *et al*., 2007). The genetic modification is the most important characteristic of biotechnology; it is for the improvement of plants, animals, micro-organisms and for the benefit of humans. A huge number of modified genes have been successfully transferred into different crops (Christou, 1997; Hansen and Wright, 1999; Repellin *et al.,* 2001; Lu and Snow, 2005; Lee *et al.,* 2006; Zhoa *et al.,* 2007).In 2014, genetically modified (GM) crops were grown by 18 million farmers in 28 countries on a total surface of 181.5 million hectares (Luchte*al.,* 2015).Traits which have been introduced in plants include high level of protein content, unique nutritional compounds (Gura, 1999; Hasler, 2000), disease and insect resistance (Datta *et al*., 2002; Bock, 2007), resistance against viruses (Shephered *et al.,* 2007; Vanderschuren *et al.,* 2007), resistance against herbicides (Lutz *et al.,* 2001; Toyama *et al.,* 2003) as well as salt and drought tolerance (Bahieldin *et al.,* 2005; Tang *et al.,* 2006). Despite all the advantages of GM crops, biosafety assessment still remains at the core of new developments (Shahid *et al.,* 2016).

The current study was conducted on Bt sugarcane to assess possible risk factors associated with the use of transgenic crop. The feeding trial was of 120 days. The impact of genetically modified sugarcane that contains transgene from *Bacillus thuringiensis* on chicken was evaluated.With the advent of Bt crops, the use of insecticide has been reduced significantly. This reduction benefits not only human but also non-target insect populations. The Bt crops require fewer insecticide treatments per year as compared to non-Bt crops where 5-12 insecticide treatments are needed (Azam*et al*., 2013).Simultaneously, insect biodiversity could also be enhanced by the reduction of broad-spectrum insecticides, and would allow natural predator versus prey interactions to occur enhancing pest control. Beside enormous benefits of GM crops, these have been considered a potential threat to environment and human health. Therefore, assessment of any potential risk, the GM crop may pose needs to be investigated.

In the current study, we have described the various aspects of risks associated with Bt sugarcane and its assessment in perspective of safe release of GM sugarcane. Four assorted groups of chicken were fed on GM sugarcane for 120 days followed by assessment of any possible insertion of the transgene in feeding animal through PCR assay. In terms of biosafety assessment, the identification at molecular level the difference between genetically modified crop and its counterpart is mandatory and prerequisite (Szenasi *et al.,* 2014).RT-PCR was carried out to evaluate the persistence of GM transcripts in different tissues of the test animals (chicken) by mixing Btsugarcane meal in their diet.No amplification of Cry1Ac transgene was observed in blood and in tissues of feeding animals depicting that no unintended effect of the genetic modifications has taken place in the GM feeding animal. Our results also demonstrated that the chicken feeding on transgenic sugarcane has no significant difference in weight when compared with the birds fed on commercial diet indicating that the feeding content of the test diet were normal and doesn't cause any difference among animals in assorted groups in any form. Similar results were obtained by Taylor *et al.* (2007).Biochemical analysis of the blood sampled from test animals depicted that no significant difference in the ALT, AST, ALP, Creatine, and Urea exist between four assorted groups. The genes introduced into the plant may result in the synthesis of new substances that can be novel in the context of the genetically modified crop and may be toxic for the feeding animal. Similar findings were reported by Hammond *et al.* (2004).Tony *et al.* (2003) conducted a similar experiment where chickens were dissected and weight of their organs was measured as proposed by Hammond *et al.* (2004). Stability of foreign DNA in feed is the prerequisite for its presence in vital organs of the subjected animal. In our findings, histologically no difference in cellular architecture of vital organs in all four assorted groups was observed. These findings comply with Marinucci *et al.* (2008) who have obtained the similar results on sheep. Conclusively, CEMB

developed GM sugarcane is safe for consumption as supported with current feeding trial.

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