

RISK ASSESSMENT OF TRANSGENIC COTTON HARBORING Bt AND GLYPHOSATE RESISTANCE GENE ON FISH (*Labeo rohita*)

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ABSTRACT

Assessment of potential risk of transgenic *Bt* protein on Rohu fish (*Labeo rohita*) was done. This sensitive vertebrate is an important part of the food chain and is commonly eaten in Pakistan. Comparative analyses on *Labeo rohita* were performed in three groups *i.e.* fish fed with *Bt* diet, non *Bt* diet and glyphosate resistant protein containing diet to evaluate the differences in their morphological, histological, biochemical and molecular characteristics. No noticeable difference was observed in morphologies as well as biochemical activities of organs. The analysis include glucose, ascorbic acid, total lipids, cortisol, glycogen, phospholipids, cholesterol, triglycerides and free fatty acid test. Structural and morphological bone analyses did not show any deformities, holes and unusual bends which concluded non-significant effect of modified diet. The presence of *Cry* and *Cp4EPSPS* protein in experimental samples was confirmed through ELISA. The biochemical and molecular analysis also confirmed that, there was no negative effect of *Cry* and *Cp4EPSPS* protein on fish.

Key words: Bt Cotton, Glyphosate, *Labeo rohita*, resistance, transgenic,

INTRODUCTION

Genetically modified crops are produced by adding one or more genes to a plant genome, by method called 'transformation' and plants created are called as GMOs. Most common modes of transformation are particle bombardment and horizontal gene transfer by *Agrobacterium tumefaciens*, a soil bacterium (Foster *et al.*, 2006). GMOs are given much attention in public discussions about their safety for final consumer and non-target organisms (Australian Science Media Center, 2007). There are many reasons which lead to development of transgenic plants or their products like longer shelf life, herbicide resistance, disease and pest resistance, abiotic stress resistances and nutritional improvement (Bezi *et al.*, 2018). *Flavr Savr* tomato was first ever transgenic crop commercially approved for sale in US in 1994. Before this hybrids have been produced for crop improvement by conventional breeding methods (Elizabeth Finkel Science, 2008). Even wheat is considered to be a natural transgenic plant emerged from diverse parental combinations with time (Sarah *et al.*, 2005).

After the commercialization of *Flavr Savr*, other crops like GM-soya and GM-corn started to be cultivated outdoors on large scales in open environment, especially in USA (Australian Science Media Center, 2008). *Bacillus thuringiensis* (*Bt*) corn brought revolution in the mid-

1990s, and planting of GM crops increased dramatically throughout the world (Cannon *et al.* 2000).

With the introduction of GM crops for commercial production, an interest developed how transgenic crops and relevant agricultural practices may affect the environment (Dale *et al.* 2002). Many questions have been raised about potential impacts of GMOs on biodiversity, non-target organisms and soil microbiota.

So the need emerges to ensure the biosafety assessment of transgenic crops on environment and end consumer. Several studies have been conducted in recent years to examine the safety of transgenic crops, especially with reference to their effect on the fitness of non-target organisms (Groot adduce, 2002). Many studies have been done for biosafety assessment of genetically modified crops and their fate when consumed by end user. Indirectly, the organism which directly feeds on GMOs and later become part of human diet are most important to be studied for risk assessment.

Previously Shahid *et al.* (2016) and Mahmood-ur-Rehman *et al.* (2007) reported that transgenic crops have no effect on animals and non-target insects. To see the effect of *Bt* diet on animals. Takami *et al.*, (2008) established an experiment for four weeks on F344 rat as well as Tralbalza-Marinucci *et al.*, (2008), on sheep and they did not find any type of effect of *Bt* diet on these animals.

The primary objective of the present study was to estimate the harmful effects of *Bt* and GTG protein in Rohu fish (*Labeo rohita*) on parameters like, fish weight,

size, development and growth. The main purpose of the selecting this fish for the risk assessment study, as it is very sensitive vertebrate and is an important part of the food chain and commonly eaten in Pakistan, Bangladesh, Sri Lanka and the Indian states. Rohu is a large, silver-colored fish of typical cyprinid shape, with an evidently arched head.

MATERIALS AND METHODS

This study was carried out at National Center of Excellence in Molecular Biology (CEMB), University of the Punjab. Two varieties of transgenic cotton containing *Bt* (*Cry1Ac+Cry2A*) and glyphosate tolerant (Cp4 EPSPS) genes were used in the diet of fish.

Selection of Fish: Rohu fish (*Labeo rohita*) used in this experiment were bought from Manawa Fish Farm, Lahore, Pakistan. Seventy-five fish (One-week old) were taken for these studies and divided into three groups, transgenic diet group *Bt* (*Cry1Ac+Cry2A*), EPSPS and control group. Each group contained twenty-five fish, kept in separate cemented ponds having the dimension of 4 feet wide X 4 feet long. Clean water of ponds was changed at regular interval to maintain the desired temperature and pH.

Composition of Diet: *Bt* and EPSPS transgenic cottonseeds were crushed and 25% of the crushed seed was then added to diet of fish. The basic ingredients of diet were as follow:

Fish Meal	40g
Maize Gluten	30g
Oil	15ml
Vitamin Mineral Mix	5g
Rice Polish	7.5g
Wheat Flour	25g

Note: Transgenic cotton seeds were added in the diet at the rate of 25g/100g in the in ground form.

Dissection of Fish: A single organism from each group *i.e* control and experimental (*Bt* and GTG) group were dissected at the end of experiment. All the organs were profused with Phosphate-buffered saline (PBS) and released blood was collected for further use in biochemical & molecular analysis. Using a sterilized blade, vital organs and muscles were cut down and preserved for morphological, biochemical and histological studies. Following organs were selected.

- Gallbladder
- Intestine
- Heart

Morphology of Organs: To assess the effect of *Bt* and GTG morphological variations were observed, in term of

organ structure, organ weight and maturity levels of organs.

Histological Analysis of Organs: At the end of study period, the following organs and tissue (*i.e.* liver, kidney and intestine) were carefully removed for histopathological examination. Before processing for hematoxylin and eosin stained sections, organs and tissues were rinsed with saline solution and immediately placed in 10% formalin.

Biochemical Tests: Blood, Bone and muscles samples were selected from twelve fish in each groups for different biochemical analyses *i.e* ascorbic acid, glucose, cortisol, glycogen, free fatty acids, total lipid, phospholipids, cholesterol and triglycerides in order to check the efficacy of vital organ function after being fed on transgenic diet.

Sampling and Analysis: Syringes rinsed with 27% ethylenediaminetetraacetic acid (EDTA) were used to draw blood samples from caudal vein. Samples were immediately transferred to a test tube containing EDTA as anticoagulant. Blood samples (25 mL) were collected from twelve fish in each groups for the estimation of blood glucose and rest was centrifuged to separate plasma.

MOLECULAR ANALYSIS

Protein Extraction: The blood and fish muscle cells was taken in 1.5 ml tube and centrifuge at 13000 rpm for 20 minutes. Then blood serum was collected in separate 1.5 ml tube and used as protein sample. Serum was stored at 4 °C. After dissection, muscles and intestine were separated and washed in sterile distilled water and stored at 4 °C after adding 600 µl of Protein Extraction Buffer. [Tris-buffered saline (TBS; 10×, pH 7.5), Ethylenediaminetetraacetic acid (EDTA; 0.5M), Nonidet P-40 (NP-40;10%), H₂O]

Dipstick Assay: The Agdia Kit for *Cry1Ac* (Cat # 00008) and *Cry2A* (Cat # 00028) was used for the detection of *Cry1Ac* and *Cry2A* in the fish samples. 100 µl of each sample (*i.e* diet blood and muscle protein sample) was taken and stripped against *Cry1Ac* and *Cry2A* antibodies. The strips were dipped in each sample for 30 minutes till the band appeared.

Enzyme Linked Immune Sorbent Assay (ELISA) of Tissue, Blood and Diet Samples: 50 µl of *Cry1Ac* enzyme conjugate was added to each well of ELISA plate. The first well contained 50µl of extraction buffer as blank. Further 50 µl of *Cry1Ab/Ac* positive control was added in second well and 50 µl of sample extract in the remaining wells. Samples were kept at 37 °C for 1-2 hours. After incubation three washings were given with 1X PBS buffer. A 100 µl substrate was added to each well. After thoroughly mixing, samples were incubated

again at 37 °C for 15 to 30 minutes. In last 100 µl of stop solution was added to each sample. Same protocol was adopted for ELISA from diet as well. Optical density of samples was taken at 450nm.

Polymerase Chain Reaction (PCR): For the PCR analysis, DNA was extracted from blood, muscles and intestine tissues of fish from the control and treated groups. The concentration and quality of DNA was quantified with 0.8% gel electrophoresis. PCR was performed at reaction volume of 20 µl containing 100 ng genomic DNA template, forward and reverse primers 50 pM each, dNTPs 200 µM, 1X PCR Buffer (50 mM KCl, 1.5mM MgCl₂ and 10mM Tris-HCl) and Taq Polymerase 1 unit. The PCR condition for the detection of Cry1Ac (459bp) and Cry2A (167 bp) is follow: 95°C for 3 minutes, 95°C for 45 seconds, 56°C for 45 seconds and 72°C for 45 seconds 72°C for 10 minute (35 cycles) and final hold at 4°C. For EPSPS (111 bp), similar condition was used except of annealing temperature in which 60°C was used. All amplifications were detected from 1.5% agarose gel electrophoresis. The primers used for PCR analysis are shown below;

5' ACAGAAGACCCTTCAATATC 3' (Cry1Ac Forward Primer)

5' GTTACCGAGTGAAGATGTAA-3' (Cry1Ac Reverse Primer)

5'-TCAACAACATCATCGCATCC-3' (Cry2A Forward primer)

5'-CAGCCTCGAGTGTTGCAGTA-3' (Cry2A Reverse primer)

5'-TATGGCTTCCGCTCAGGT-3' (EPSPS Forward primer)

5'-AGCATCTTCTCAGTGTGGTCTCT-3' (EPSPS Reverse primer)

Statistical Analysis: All data are reported in the table and figures as mean ± SEM. Data analyses for statistically significant differences were done by one-way analysis of variance (ANOVA) and P value < 0.05 was considered significant. Graph Pad Prism version 7 was used for demonstration.

RESULTS

Fish Weight: The weight of randomly selected fish (12) of both experimental group (Cry1Ac/Cry2A and EPSPS gene) and control groups was measured after every seven days' interval by digital balance. The average weight of the fish in their respective groups is comparable throughout the study periods. Although, some differences were calculated in the mean values for the fish in both control and experimental groups, however, such differences were only numerical but not statistically significant (P<0.05). The examined weight was expressed in gram (g) due to the smaller size of the fish (Figure 1).

Morphology of Organs: The morphology of vital organs such as intestine and gallbladder was examined after

dissection. No significant changes were observed in color, texture, organ sclerosis and general appearance of organs. Bones of the member from each group were analysed which showed no deformity.

Histology of Organs: Thin sections of tissue from kidney (Figure 2A), intestine (Figure 2B) and liver (Figure 2C) were studied by Haematoxylin and eosin staining protocol. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. Eosin is strongly absorbed by red blood cells, coloring them bright red. After H & E staining the organs, no difference in the cellular architecture of organs of both groups was observed.

Biochemical Tests: To measure the quantity or activity of specific enzymes or protein in a blood or other tissue sample various biochemical tests were performed from serum of randomly selected fish (12) from each groups. The overall results are presented in (Table 1), all values are expressed as mean ± standard error. The results of the examined biochemical parameters did not show any treatment related effects in all the groups. All the values were comparable despite the minor differences detected in the mean values, nevertheless, such differences were only numerically but not statistically different (P<0.05).

Molecular analysis: In order to access the possible transfer/translocation of the Cry1Ac, Cry2A, EPSPS genes or their expressed proteins in the tissue of fish, molecular analysis like PCR, ELISA and dipstick analysis were performed.

Polymerase Chain Reaction: To trace the possible transfer/integration of Cry1Ac, Cry2A, EPSPS, PCR was performed from genomic DNA samples extracted from the tissues (Blood, muscles and intestine) of fish.

Similarly, the presence of Cry1Ac, Cry2A and EPSPS was confirmed in the formulated diet used for this study (Figure 3). Following the PCR analysis, none of the Cry1Ac, Cry2A or EPSPS gene was detected in the analyzed samples of (Blood, muscles and intestine). However as expected, the presence of Cry1Ac, Cry2A or EPSPS was recovered from the DNA samples extracted from the formulated diet used as the positive control.

Dipstick assay for detection of residual Bt protein (Cry1Ac and Cry2A): Dipstick assay was done to detect the presence of Bt proteins in the formulated diet, blood and muscle protein sample of the fish. There was no trace of Cry1Ac or Cry2A detected in all the analyzed fish samples except in the positive control sample. Conversely, the presence Cry1Ac or Cry2A was confirmed in the diet samples as well as in the positive control samples (Figure 4).

Detection of residual target protein from the fish tissue: Commercially available ELISA kits, Envirologix kit (catalog # AP003) for Cry1Ac, Envirologix kit (catalog # AP005) for Cry2A and Envirologix kit (catalog # AP010) for CP4EPSPS were used to detect the target Cry1Ac, Cry2A or CP4 EPSPS proteins in the protein

samples extracted from fish tissue (muscles and blood serum). For this assay, serum was diluted to 1:10 concentration. Results confirmed absence of Cry1Ac, Cry2A or EPSPS in all the tissues samples except in the positive control sample as shown in Figure 5.

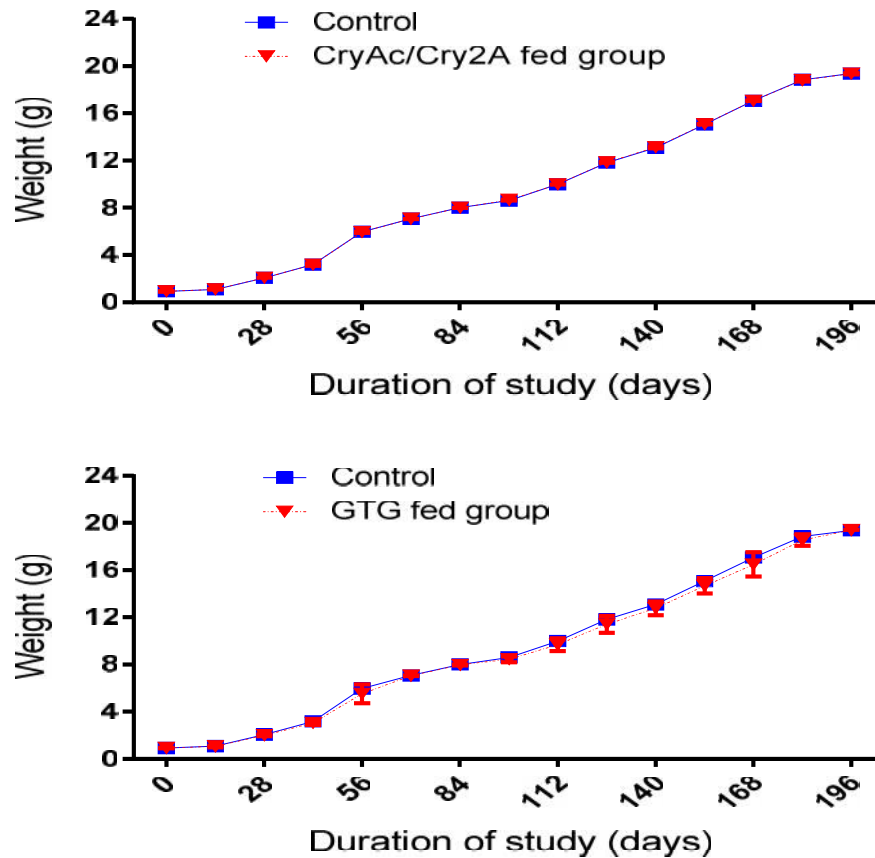


Figure 1. Comparison in weight of control and transgenic fish groups

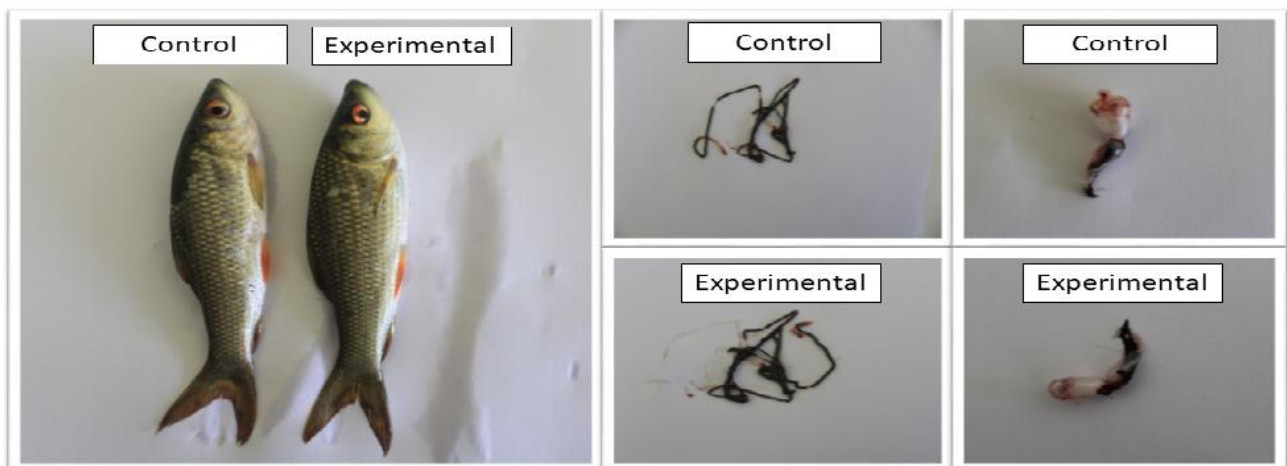
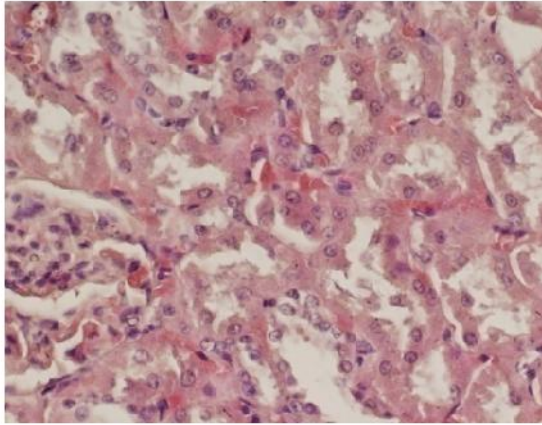
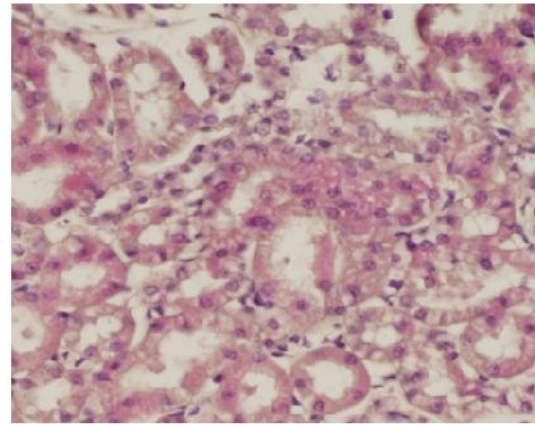


Figure 2. Morphological analyses of vital organs (heart, intestine, and Gallbladder) of control and Experimental (transgenic) groups

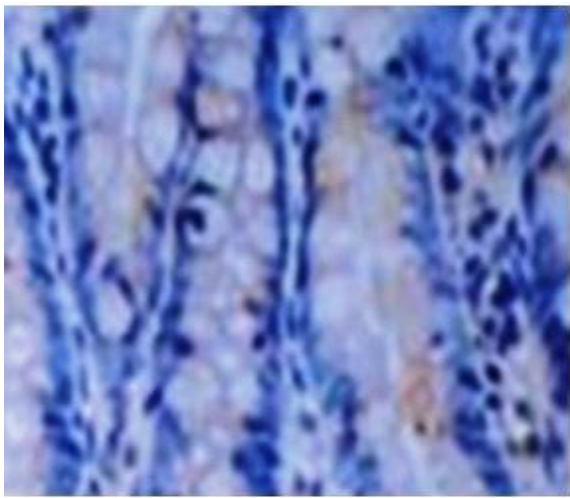


Experimental

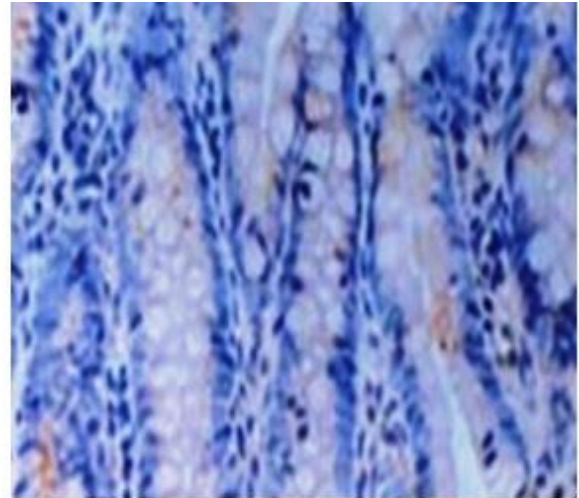


Control

Figure 3A. Comparison between cellular architecture of Kidney of experimental and control Fish.

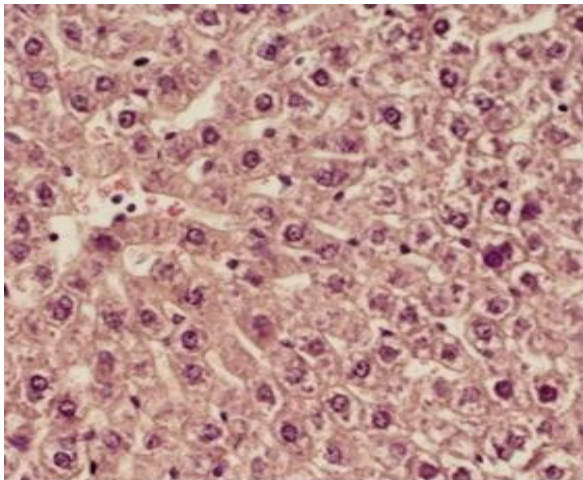


Experimental

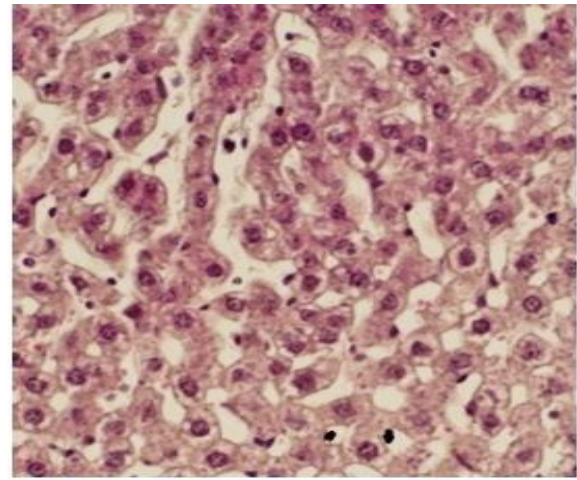


Control

Figure 3B. Comparison between cellular architecture of intestine of experimental and control Fish.



Experimental



Control

Figure 3C. Comparison between cellular architecture of liver of experimental and control Fish.

Table 1. Effects of transgenic diets on fish biochemical parameters

Parameters	Feeding groups			P- values
	Control	Cry1Ac/Cry2a	EPSPS fed	
Glucose (mg 100 ml ⁻¹)	16.99 ± 0.59 a	16.67±0.57 a	17.00±0.62 a	0.9049
Cortisol (ng ml ⁻¹)	21.74 ± 0.77 a	21.52 ± 0.73 a	21.58 ± 0.81 a	0.9786
Glycogen (mg g ⁻¹ wet tissue)	10.61 ± 0.48a	10.39 ± 0.53a	10.59 ± 0.50a	0.944
Ascorbic acid (mg g ⁻¹ wet tissue)	241.51 ± 5.51a	239.99 ± 5.14a	240.41± 5.21a	0.979
Total lipid (mg g ⁻¹ wet tissue)	65.86 ± 1.62a	66.40 ± 1.33a	65.77± 1.61a	0.952
Phospholipid (mg g ⁻¹ wet tissue)	16.84± 0.23a	16.62 ± 0.25a	16.80 ± 0.23a	0.788
Cholesterol (mg g ⁻¹ wet tissue)	9.82 ± 0.45a	9.80 ± 0.47a	9.84 ± 0.40a	0.998
cTri and dFA (mg g ⁻¹ wet tissue)	48.06 ± 1.45a	48.22 ± 1.56a	48.15 ± 1.53a	0.997

Values in the table are mean ±SEM. ^cTri = Triglycerides, ^dFA = free fatty acids

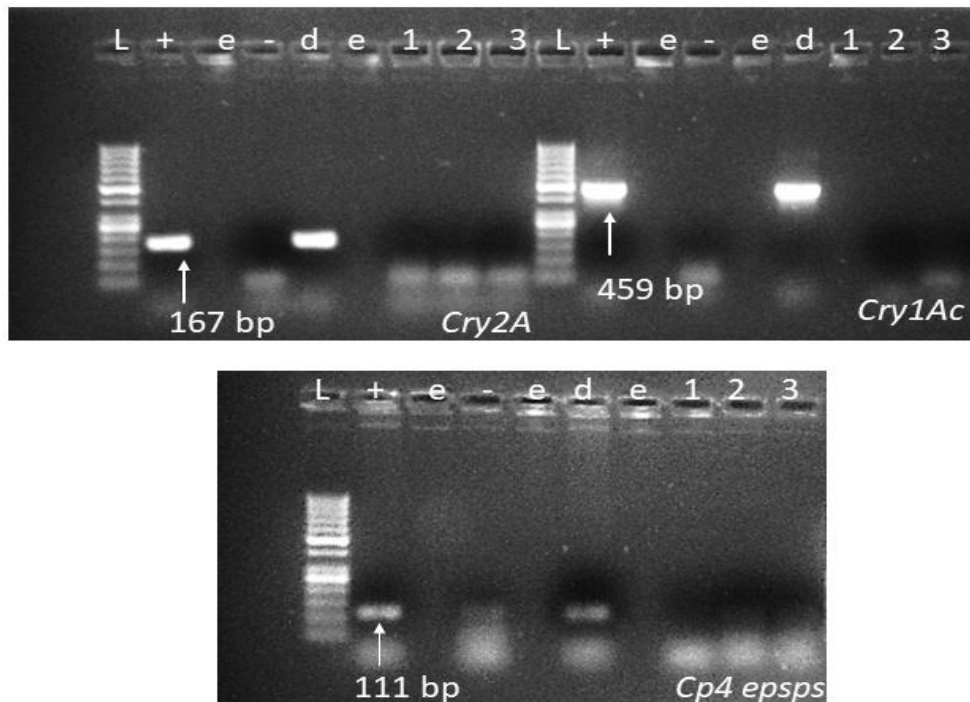


Figure 3. PCR result for the detection of Cry1Ac, Cry2A and Cp4epsps in the diet and fish samples. L; 50 bp ladder, +; positive control, e; empty well, -; negative control, d; diet sample, 1,2 and 3; represent the samples from blood, muscles and intestine respectively.

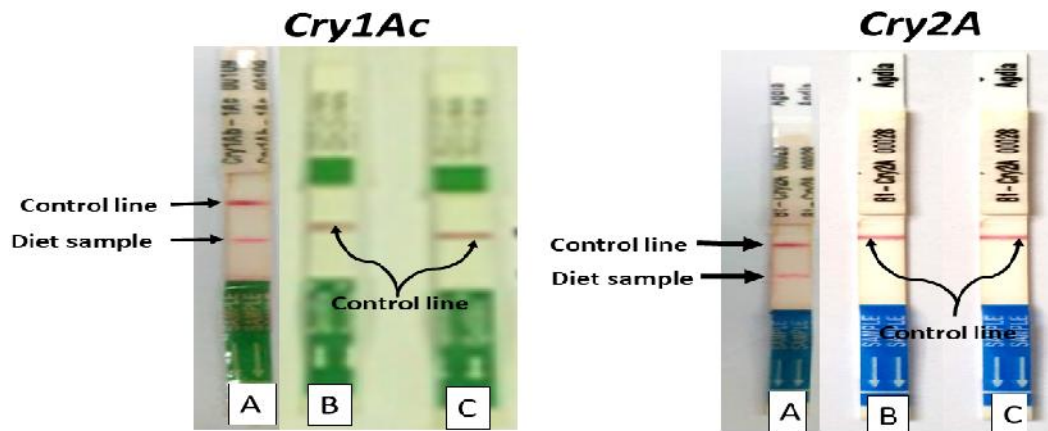


Figure 4. Dipstick assay for the detection of Cry1Ac and Cry2A from the diet (A), blood (B) and muscle tissues (C).

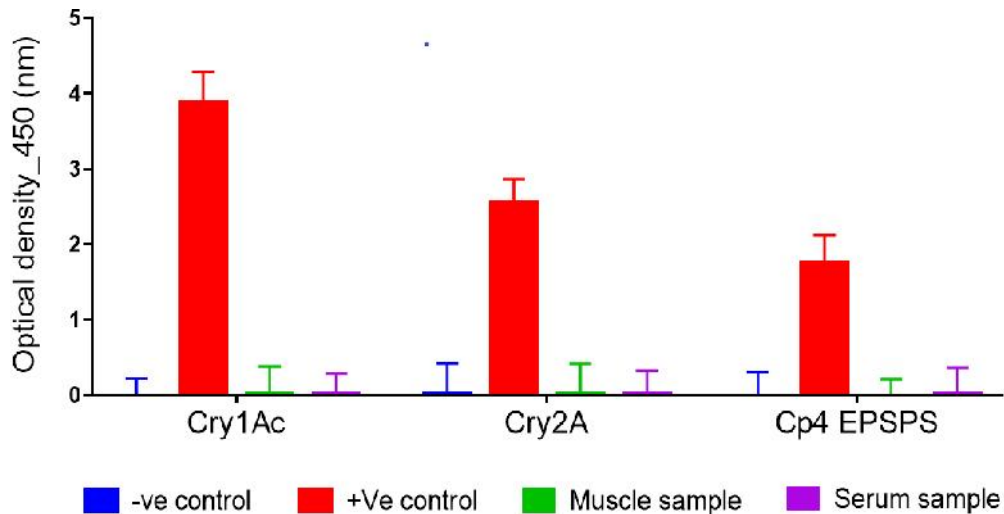


Figure 5. ELISA Results of protein samples extracted from fish.

DISCUSSION

The primary objective of this study was to estimate the harmful effects of Cry1Ac, Cry2A or CP4 EPSPS protein on Rohu fish on different traits like fish weight, size, development and growth. For assessing the effect of Cry1Ac, Cry2A or CP4 EPSPS, the Rohu fish was grouped in three different groups which were fed on non-transgenic, Bt (Bt protein obtained from Bt cotton seed) and EPSPS protein containing diet. Normal increase in weight was observed in the groups, and no marked differences were noticed in growth and performance between fish fed the normal and GM-based diet in the present study. This result is in agreement with Hammond *et al.*, (1996) and Sanden *et al.*, (2004) who reported catfish and Atlantic salmon fed either glyphosate-tolerant or unmodified soybean diet showed normal growth. Similarly, Sissener *et al.*, (2010), observed no significant changes in the growth of Zebrafish (*Danio rerio*) when Bt maize was supplemented in their diet for 20 days. Consistently, Chainark *et al.*, (2006) also reported no significant difference in growth and feed performance when rainbow trout were fed GM or non-GM soybean for 12th week.

Histopathological examination is an effective laboratory technique in cases where other diagnostic methods fail. The technique on the other hand, remain indispensable in pathomorphological evaluation of side effects of vaccines, drugs, and chemical compounds (Reichert *et al.*, 2012). In this study, histopathological examination of the fish vital organs tissue (Liver, kidney and intestine) was performed, and the result of the examination showed no significant differences between the control animals fed diets containing no genetically modified feeds and animals fed genetically modified feeds. The results of this study confirm the findings of other authors on the lack of harmful effects of GM

products in feed materials on animal health. Such conclusion predominates in the majority of publications (Jennings *et al.*, 2003. Kulikov 2005, Kwiatek *et al.*, 2008).

Serum biochemical test are very crucial for clinical investigation of several constituents of animal bodies (Salisu *et al.*, 2018). In this study, various serum biochemical parameters were measured in response to GM feed consumption. No considerable changes were detected in all the tested serum biochemical parameters of both the control and experimental groups of fish. The results for serum biochemical test obtained in this study obviously revealed that, exposing the fish to dietary GM cottonseeds for 196 days did not adversely affect the function of their vital organs such as the liver, kidneys and heart. These results are consistent with the previous reports, in which no significant alterations were found in various biochemical parameters examined when GM feed ingredients were supplemented in the diet of a number of animal models including the fish (Salisu *et al.*, 2018, Sissener *et al.*, 2010, Tudisco *et al.*, 2006, Yonemochi *et al.*, 2003).

Currently, the detection and fate of transgenic DNA and/or proteins in animal feed and their tissues has been emphasized as an essential issue in the ongoing debate over the use of transgenic crops in animals feed as well as human food products (Nadal *et al.*, 2018). Humans and animals are continuously exposed to diverse sources of exogenous DNA as part of their diet. DNA and proteins are constituents of almost all feedstuffs and foods. Scientist have reported that both human and animals have the historic ingestion of DNA and proteins of plant and animal origin, without any real evidence of related health problems (Beever *et al.*, 2003). In this study, the possible uptake/translocation of the gene fragment or expressed proteins from the GM based diet to the fish tissues (Blood, muscles and intestine) was

monitored via PCR and ELISA. There was no trace of Cry1Ac, Cry2A or CP4 EPSPS gene fragment detected in all the DNA samples extracted from blood, muscles and intestine during the PCR analysis, except in positive samples PCR of fish diet which confirmed the presence of Cry1Ac, Cry2A or EPSPS gene fragment in the transgenic diet. Correspondingly, none of the Bt or EPSPS protein was detected in protein samples extracted from the fish tissues during the ELISA assay except in positive samples. The absence of feed-derived DNA in the PCR analyzed tissues of fish clearly indicated that the dietary DNA completely degraded in the gastro intestinal track of the fish due to enzymatic activities. The outcomes of this study are in accordance with report of Sanden *et al.*, (2011), who found no trace of transgenic DNA fragment in the DNA samples extracted from the intestinal tract of Atlantic salmon fed either genetically modified (GM) or conventional (non-GM) soybeans for six months. Additionally, Sanden *et al.*, (2004) reported that no transgenic promoter soy DNA fragment (195 bp) was found in muscle of Atlantic salmon.

On the other hands, contradictory results in fish studies on GM and non-GM SBM diets were reported; for instance, Nielsen *et al.*, (2005) reported that all three DNA targets, CaMV 35S, Roundup Ready soybean and Cry1 A(b), were detected in kidney, liver and blood of Atlantic salmon up to 64 h after force-feeding a diet containing amplified DNA fragments. Similarly, Chainark *et al.*, (2006) reported that the transgenic DNA fragment could be amplified in the whole content GI tract, kidney, leucocytes, and muscle tissue Fish (rainbow trout) fed transgenic soybean meal.

Conclusion: *Bt* cotton risk assessment was done using different biochemical tests and molecular techniques. The results showed no significant differences in the control diet fed fish and transgenic diet fish. But further studies are required for long term impact study.

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Authors' contributions: Tahira Haider and Tahir Rehman Samiullah conducted the experimental work; Muhammad Azam Ali and Kamran Shahzad Bajwa helped in Molecular analysis, experimental design and material collection. Ibrahim Bala Salisu did statistical analysis, AQ Rao reviewed the manuscript, Ahmad Ali Shahid and T. Husnain helped in guiding and conceiving the study, conducting it and writing the manuscript. All authors read and approved the final manuscript.

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