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Evaluation of potential impact of GM cotton on Chicks

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Abstract

A feeding study was conducted for 120 days on chicks (*Gallus gallus* L.) to evaluate effect of two varieties of transgenic cotton containing Bt (CEMB-Cry1Ac +Cry2A) and GTG gene (EPSPS enzyme) on growth, egg productivity and clinical chemistry of blood, serum protein and histology of organs. A total of 90- and 15-days old layer chicks were taken and divided in three groups each with three replicates. Two types of diets were given to chicks i.e. transgenic cotton with Cry1Ac+Cry2A gene, cotton leaves containing GTG gene and non-transgenic cotton served as control. Birds weighed at fifteen days intervals showed no significance difference in their weight. Egg production rate was significantly different in the different treatments. Clinical chemistry of blood, serum protein and histological analysis of organs (heart, liver, spleen and intestine) showed no significant difference in the transgenic diet and control diet fed animals.

Keywords: Bt gene, Cry protein, GTGene, New castle Disease.

Introduction

Population explosion is the major concern now-a-days in the world so there is dire need to develop certain strategies to overcome the increasing population food demands especially in developing countries (Jennings *et al*., 2003). Agriculture is the basic field in which effort is applied to change output results. In the past traditional breeding was done to improve the yield of the crops, with rapid increase in population it became necessary to device certain methods and techniques to overcome the problems of hunger, malnutrition, insect pest attack, increasing herbicide use, salinity of soil and tolerance of crops to adverse conditions. Keeping all in mind, scientists devised certain procedures to improve the quality of crops by utilizing genetic engineering (McNaughton, 2004).

Transgenic crops are being used for the welfare of mankind since three decades, these crops are providing benefits in improving both quality and quantity of the product. The first modern recombinant crop was the Flavr Savr tomato was approved in 1994 for sale in the US, , due to a longer shelf life (Redenbaugh *et al.*, 1994; Rehout *et al*., 2009). Golden rice is a good example fulfilling the deficiency of vitamin A as its deficiency cause blindness in third world so this variety provides a cost free solution for the deficiency of carotenoids (Ye *et al.,* 2000). Where there are many benefits there are some risks, which are to be taken into account that either these crops are beneficial for human or animal use or not. Therefore, safety evaluation of genetically modified foods is performed to identify and characterize any unexpected changes which could affect the safety or nutritional status of the modified organism. Therefore, methods are under development to identify unintended effects through DNA sequence and analysis mRNA/protein, or analysis of problems of secondary metabolites (Noteborn and Lommen, 1998).

Scheideler *et al*. (2006) concluded the transfer of DNA fragments into the body is a normal process that takes place constantly, while Aeschbacher *et al.* (2005) noticed no recombinant DNA in the PCR products. Sharma *et al.* (2007) investigation showed that Cp4Epsp DNA of canola degraded rapidly and PCR analysis showed none of Cp4 Epsp fragments in human intestine. No differences were observed regarding performance, reproductive traits, hematological parameters, antioxidant defenses, lymphocyte proliferative capacity, phagocytosis and intracellular killing of macrophages, and ruminal microbial population characteristics between control and genetically modified (GM) maize-fed animals (Marinucci *et al.*, 2008; Sauvé and Brake, 2010). In this study, an attempt was made to evaluate the biosafety effects of transgenic cotton on chicks, and useful or harmful effects on chick's health using molecular and biochemical analyses.

Materials and Methods

Experimental design

Fifteen days old chicks were selected and randomly assorted in three groups of 136-day feeding trial. Ninety chicks were randomly distributed in three groups each containing 30 birds, each group was further divided in three replicates containing ten birds each.

Diet composition

Genetically modified cotton crop was used with Bt genes (Cry1Ac+Cry2A) and GTG gene (EPSP) that developed at Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan. The amount of Cry1Ac+Cry2A protein in cotton variety was confirmed by ELISA, and protein present in the grain was 0.24 g seed^{-1}. Transgenic and non-transgenic seeds were processed (delinted) and stored. Three types of diets were formulated containing transgenic cotton with Cry1Ac+Cry2A gene, cotton leaves containing GTG gene, non-transgenic cotton as control.

Housing and vaccination of chicks

Chicks were housed on wooden shavings and continuous supply of water and feed was provided. The temperature of the room was maintained initially at 35 °C and afterwards 25 °C was maintained for rest of study (Elangovan *et al.*, 2006). At day one, the chicks were vaccinated with New castle Disease (ND),, after 3-weeks these were again vaccinated with ND (inactive) and at the age of one month vaccination was given against infectious bursal disease (Giambrone and Clay, 1986).

Analysis of blood samples and weight

Weighed of transgenic and non-transgenic diet fed animals were taken at the interval of fifteen days for four months. Blood samples were also collected from each chick at intervals of 15 days to check the presence of *Cry1Ac* protein. About 500 µL blood was taken from the brachial vein (under the wing) using 1cc syringe and stored in EDTA vacutainer (Rossi *et al.*, 2005). Blood samples were then centrifuged at 13000 rpm for 15min at 4 °C, and serum was pipetted out for dipstick, ELISA and SDS-PAGE analysis (Chansawang *et al*., 2003).

Egg production and protein extraction from eggs

Eggs produced by control and experimental groups were recorded daily. Eggs were duly labeled and data were recorded (Swiatkiewiez *et al*., 2009).

Homogenized mixture (500 uL) of chick's egg was dispensed into 1.5 mL tube tubes and about 300 µL ice-cold extraction buffer (10 mM Tris HCl, pH 7.5, 40 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mg mL^{-1} DTT and 1 mM PMSF) was added. Tubes were kept on ice for twenty minutes then centrifuged at 4 °C for 5 minutes at 13,000 rpm. Supernatant was transfer to new 1.5 mL tube containing extracted protein (Watanabe *et al.*, 2005).

Quantification of Cry1Ac protein and roundup ready glyphosate protein

Dipsticks were used for quantification of Cry1Ac and GTG protein from blood samples of chicks after feeding transgenic cotton. Serum extracted from the blood samples was diluted in PBS at different ratios and these were tested by Cry1Ac and glyphosate specific Dipsticks. Similarly the egg extract was also analyzed for this purpose and each sample was dispensed in a labeled 1.5 mL tube and then the dipsticks were carefully placed in the sample for 30 minutes afterwards removed, dried and then photo was captured.

Enzyme linked immunosorbent assay (ELISA)

ELISA was performed against Cry1Ac, Cry2A and glyphosate tolerant gene was performed by using Envirologix Quantiplate kit. Serum protein samples were diluted in phosphate buffer saline (PBS) and then subjected to ELISA.

ELISA for Cry1Ac and roundup ready cotton

For ELISA, 50 uL of Cry1Ac (Bt) and GTG (roundup ready) enzyme conjugate were added to each well of ELISA plate (Envirologix). Wells were duly labeled and Cry1Ac and GTG were added in the wells, respectively. Chick's serum samples as well as negative control (water) were added to respective wells, contents of the well were thoroughly mixed by vigorously vortexing the plate for 20-30 seconds, plate was covered and then incubated at 37 ºC for 45 minutes. After incubation contents of the wells were shaken into sink, wells were flooded with 1X PBS for washing. Washing was done thrice. Then 100 µL of substrate solution was added in the wells and contents were thoroughly mixed. At the end 100 uL of stop solution (1N HCl) was added, and contents were mixed well. The plate was read on ELISA-reader (SPEC-Tram ax plus 384) (Rao *et al.*, 2011).

SDS-PAGE

SDS-PAGE was carried out for quantitative measurement of chicks serum protein according to the method of Laemmli (1970) with some modifications. About 20 µg of extracted protein from chicks was mixed with 1x loading dye and samples were denatured by heat shock in boiling water bath for 10 minutes. Analysis by SDS-PAGE was carried out by using 1 mm thick macro gels (10% resolving, and 4% stacking). Protein samples were loaded in each well and then it was run in mini Bio-Rad gel apparatus at 50 V. The gel was then stained with Commassie stain (0.25% Commassie brilliant blue R 250, 45.5% methanol and 9% glacial acetic acid) for 2 hours and the gel was destained by using destaining solution (25% methanol, 7% glacial acetic acid). The photograph of gel was captured in gel documentation system.

Clinical chemistry of blood

Two birds per group were slaughtered at the end of feeding period, blood was collected in EDTA vacutainer and samples were then sent to Zenat medical laboratories Lahore, for different tests as urea, alanine transferase, aspartate transferase, alkaline phosphatase, creatinine, and bilirubin in order to check the efficacy of vital organs function.

Histological study

Histological studies of vital organ tissues were performed according to the protocol describe by Rossi *et al*. (2005) with some modifications. Two animals per treatment were slaughtered at the end of feeding study, organs including heart, liver, spleen and intestine were cut using sterilized surgical blade organs were washed using 1X PBS, heart was perfused by cardiac puncture. These organs were carefully cut into specific small pieces and were placed in 4% paraformaldehyde solution for fixation overnight at room temperature with constant shaking in 15 mL tubes. Organs were cut in transverse sections. While heart pieces were cut in the form of rings. One of the finest rings was selected. On the other hand liver pieces were selected from the anterior lobe.

Processing and paraffin block preparation of the tissues

Dehydration is the next step after fixing tissues in 4% paraformaldehyde solution, for this purpose 70%, 80%, 90% and 100% alcohol grades were prepared and tissue sections were treated for 40 to 45 minutes in each grade with constant shaking. Tissues sections were then treated with xylene for complete dehydration for 30 minutes.

For block preparation paraffin wax was melted in 50 mL tube, afterwards tissues sections were immersed in molten was for 30 minutes at 72 °C, wax infiltration was done twice. After paraffin infiltration, paraffin block of tissue were prepared. For this, mould was cleaned with xylene. Molten paraffin was poured into mould. Tissue section was placed in the center of mould, avoiding any air bubble near or under the tissue section. Cassettes were labeled with name of section and date. Appropriately labeled cassette was placed over tissue in correct orientation. Molten paraffin was poured over cassette to fix it and paraffin was allowed to solidify at room temperature. Moulds were then placed at 4 °C for half an hour. Tissue blocks were removed from mould, wrapped in aluminium foil and stored in refrigerator at 4 °C (Scholtz *et al.,* 2009).

Preparation of tissue sections

Microtome was set at 47 ºC. Tissue blocks were placed in refrigerator before sectioning, for 30 minutes at 20 ºC. After this, paraffin embedded tissues were cut and sections were taken on glass slides, labeled and stored at room temperature.

Hematoxylene and Eosin staining of tissue sections

Hematoxyline and Eosine staining was carried out as follows. Tissue sections were treated with xylene for complete removal of paraffin wax and were passed through alcohol grades i.e., 100%, 95%, 85% and 70% for rehydration purpose for 3 minutes. Slides having tissue sections were washed with distilled water and treated with Hematoxylene for fifteen minutes. Sections were then subjected to Eosin for 1 minute. Washed the slides containing sections with running water carefully and were again treated with ethanol grades i.e., 70%, 85%, 95% and 100%, respectively by dipping for few minutes for dehydration and lastly dipped in xylene. Finally cytosine mount was added to the section and put the cover slip over sections on the slide. Slides were observed under the microscope carefully.

Results and Discussion

Efforts are being made for the estimation of transgenic gene and proteins risk hazards. In this regards feeding studies were conducted and evaluated the concerned proteins (Brake and Vlachos, 1998). Chicks were weighed after every fifteen days and weight data was analyzed using Graphpad prism software. Graph showed no

significant difference in the three groups fed with GTG, Bt mixed and control (Fig. 1). Data of the three groups was further analyzed using simple correlation. Linear regression graph plotted between days and weight of the three groups and results was showed no significant difference in the weight of the three groups. R value is useful as it gives the proportion of the variance (fluctuation) of one variable that is predictable from the other variable (Fig. 2). Snell *et al.* (2012) was also reported similar results of biosafety effects on animal after feeding on GM crops.

Egg production rate was slightly different in the GTG group as compared to Bt and control group (Fig. 3) this might be due to the difference in the age difference in the control and GTG as few chicks in control group were one week younger than GTG group chicks. Dipstick was used for the detection of Cry and GTG protein in blood samples. There was no detection of protein in serum of chicks as compared to control (Fig. 4). Results were correlated with the results reported by Aeschbacher *et al*. (2002).

ELISA results clearly showed (Fig. 5) absence of Cry1Ac/Ab or GTG protein in the serum sample (McNaughton *et al.*, 2007). SDS-PAGE revealed size of of Bt protein was 68 kilo-Dalton and that of GTG was 47.6 kilo-Dalton, The results was indicated that both Cry1Ac and GTG protein were absent in the serum samples (Fig. 6). Blood samples were analyzed through Zenat lab. for biochemical analysis the results showed no significant difference in the values of ALT, AST, urea, bilirubin, creatinine (Table 1). The same study was reported by Jacobs *et al*. (2007). Histopathological study conducted for heart, liver, intestine and spleen in the three feeding groups sections were cut and stained with Hematoxyline and Eosin stains showed no alteration in different feeding groups and tissue architecture was same for all groups as shown in Fig. 7 (Jacobs *et al*., 2008).

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Table 1: Clinical chemistry of chicks blood samples after the feeding of transgenic cotton seeds containing Bt gene (Cry1Ac and Cry2A) and GTGene**.**

Biochemical test	Group		
	GTG	BT	Control
Urea (μ mol L^{-1})	5.6	5.2	5.4
Cretinine (μ mol L^{-1})	0.05	0.09	0.08
Alanine transferase (u L^{-1})	1.8	1.1	1.7
Aspartate transferase (u L^{-1})	173.5	175.4	178.1
Alkaline phosphatase (u L^{-1})	314.0	325	318
Bilirubin (mg dL^{-1})	0.04	0.03	0.02

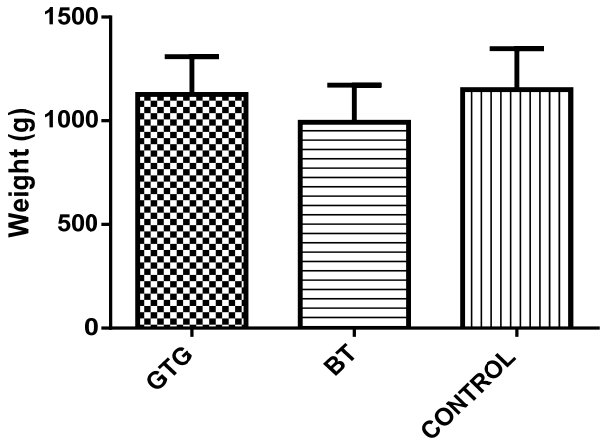

Fig. 1: Comparison of chicks weight of GTG, BT and control groups

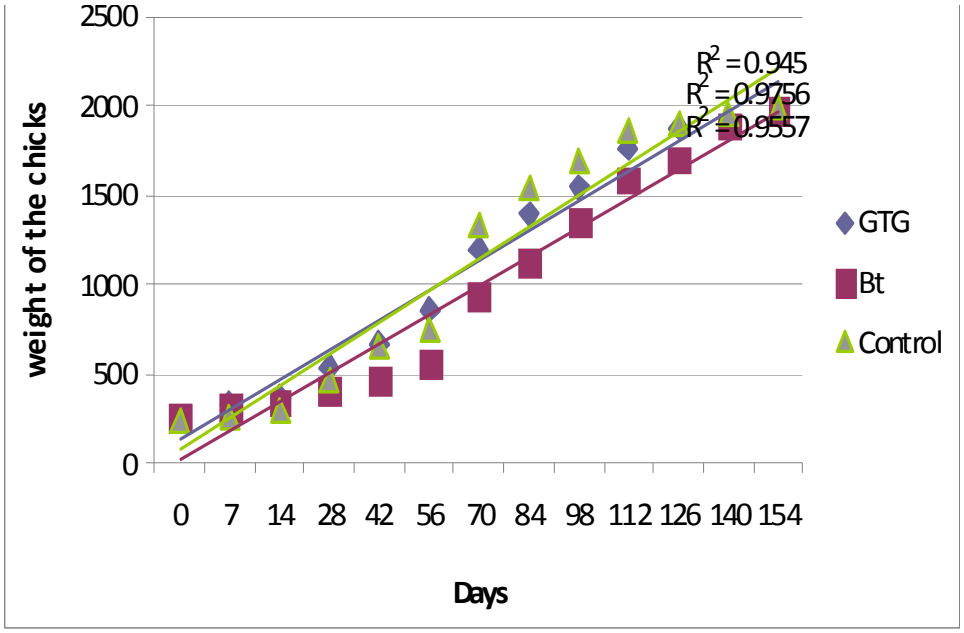

Fig. 2: The line graph plotted between the weight and days.

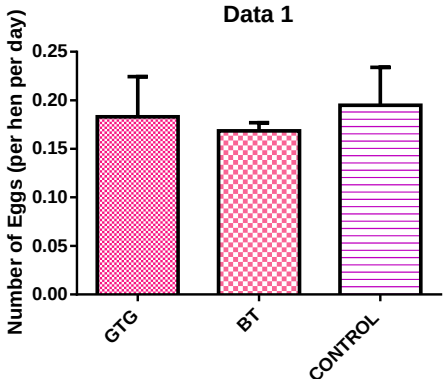

Fig. 3: Comparison of number of eggs of GTG, BT and Control groups

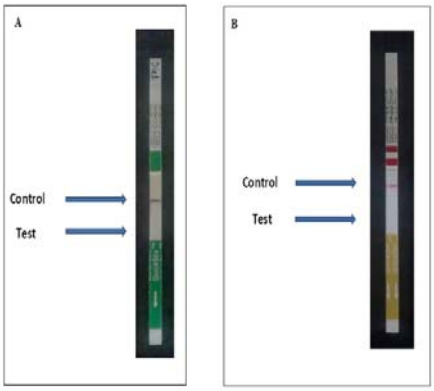

Fig. 4: Dipstick detection of protein in serum samples of chicks. (A) Cry protein; (B) detection of GTG protein.

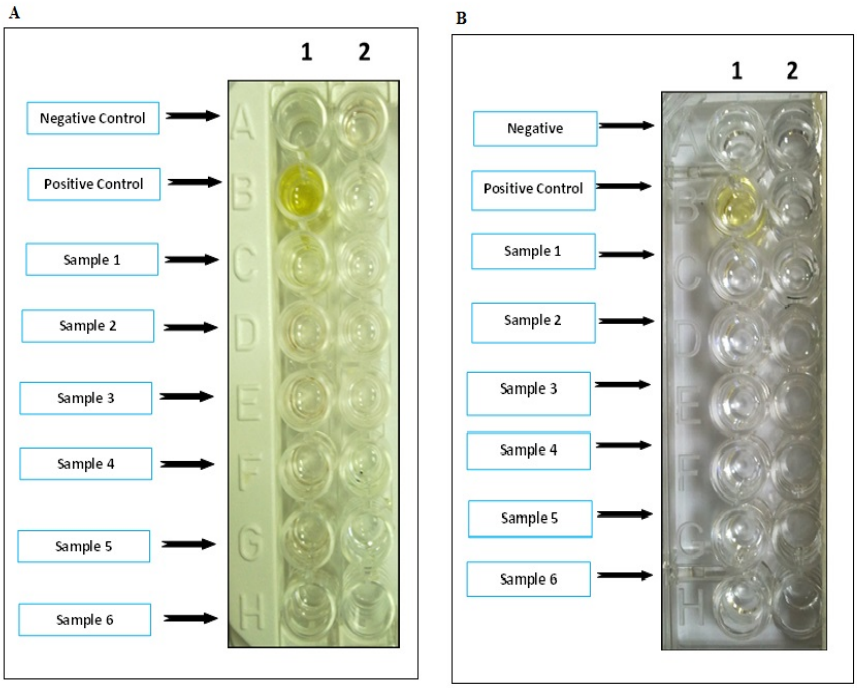

Fig. 5: ELISA for the detection of Cry1Ac and GTG protein in blood of chicks.
Negative control; Positive control; Sample 1 containing serum of the control group; and Sample 2-6 containing serum of the chicks from transgenic group.

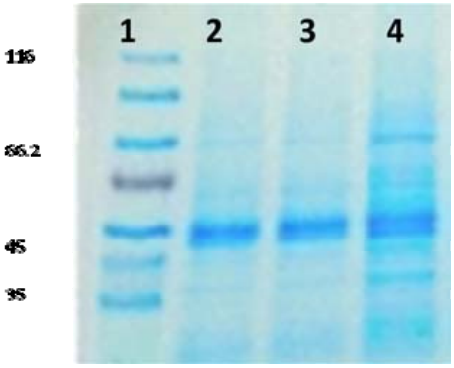

Fig. 6: SDS-PAGE of in different feeding groups of chicks.
Lane 1: Protein ladder; Lane 2: Control; Lane 3: Bt group; Lane 4: GTG group.

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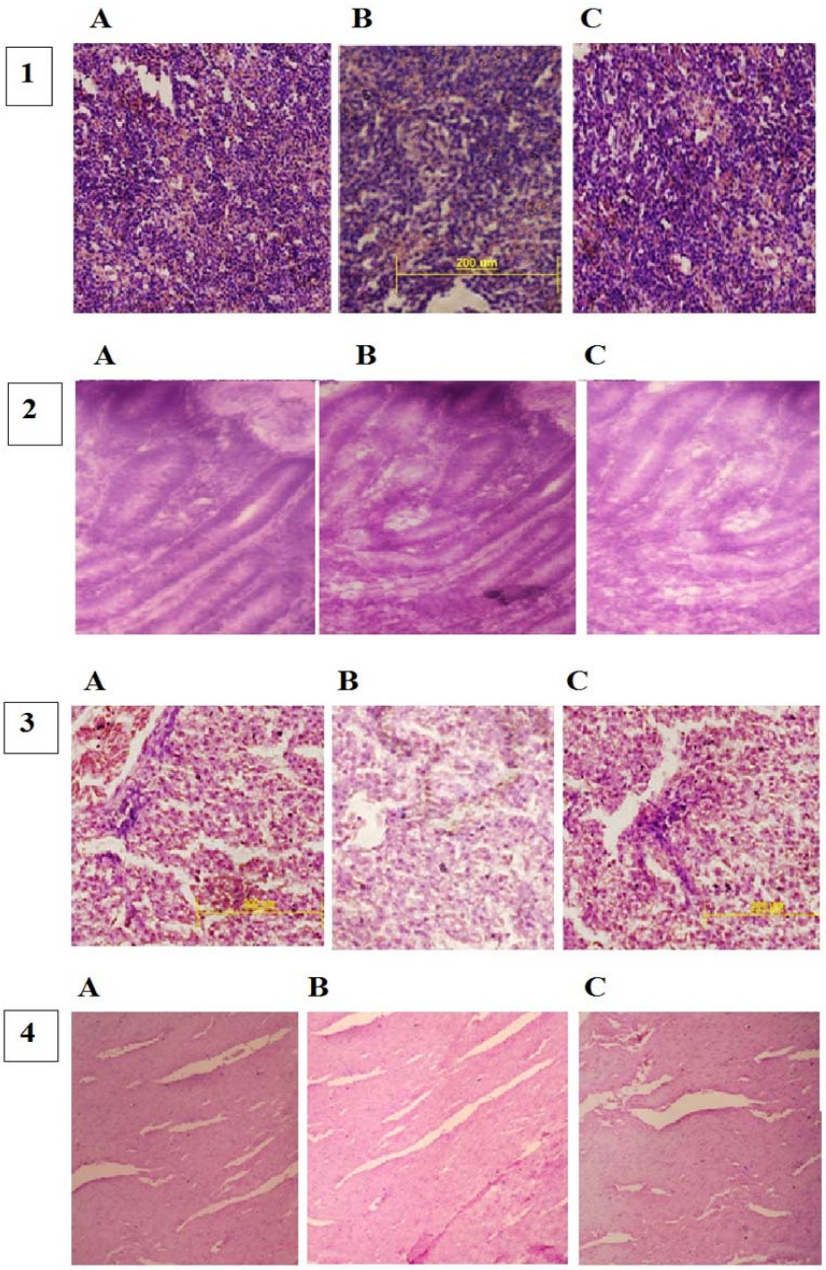

Fig. 7: Histopathological in different feeding groups of chicks.
(A) Control; (B) Bt diet fed group; (C) GTG diet fed group. Section of spleen (1) intestine section (2), liver (3) and heart (4).

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