FINAL REPORT

ON

SUBCHRONIC ORAL TOXICITY IN GOATS – 90 DAYS STUDY FOR GENETICALLY ENGINEERED BT COTTON SEEDS

Submitted to

J.K. Agri Genetics Ltd.
Hyderabad

By

Principal Investigator
U.R. Mehra

Collaborators
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Principal Insvestigator and
All Collaborators

SUBCHRONIC ORAL TOXICITY IN GOATS – 90 DAYS STUDY FOR GENETICALLY ENGINEERED SEEDS

In India, cotton is one of the most important cash crops and processed cottonseed meal is being used as animal feed. The major limitation to cotton production in India is that the crop gets damaged by insect pests attack, which leads to crop failure, loss in yield, poor cotton quality, etc. The cotton crop is damaged by about 130 species of insects of which the Lepidopteran insects are the most important. To control these pests, various types of insecticides and pesticides have widely been used. However, use of these pesticides is costly to the grower, often pose environmental hazards and have limited efficacy due to development of resistance in target pest populations.

An effective and environmentally friendly approach to control these insects and pests has been achieved through transgenic technology, where an insecticidal gene from a naturally occurring bacterium, *Bacillus thuringiensis* has been introduced into the cotton genome. This enabled production of the Cry group protein(s) in the cotton plant, which is known for its activity against lepidopteran insect pests. A popular GM cotton-popularly known as Bt cotton has been adopted broadly by growers worldwide, including India, since commercial launch in 2002. The CrylAc protein in cotton provides effective protection from feeding damage by lepidopteran insect pests, and growers using Bt cotton typically apply significantly less insecticide to control these pests, realize higher yields and achieve greater profitability as compared to conventional cotton varieties.

JK Agri Genetics Ltd have developed a new genetically modified cotton for commercial use. The transformed variety has been named as JKC 738 Bt-a *Gossypium hirustum* parental line. The introduced insect control gene from *Bacillus thuringiensis* HD 73 CrylAc (1845bp) that has insecticidal activity against Lepidopteran insect pests of cotton-mainly, American bollwarm (*Helicoverpa armigera*), Spotted bollworm (*Earias vitella*) and Pink bollwarm (*Pectinophora gossypiella*). Thus, the JK Agri Genetics Ltd has approached IVRI, Izatnagar for conducting **A 90 DAYS FEEDING STUDY WITH Bt COTTON SEED IN GOATS** with following objective, as a mandatory requirement of GOI/DBT for feed safety evaluation of the new cotton variety i.e.-**JKC 738 Bt**.

OBJECTIVE

The objective of this study was to compare the wholesomeness and feed safety of **JKC 738 Bt** (Event JK-1) cotton seeds with cotton seeds developed using non Bt versions of **JKC 738**. Cotton seeds of **JKC 738 Bt** and control cotton varieties (non Bt) was administered to goats through the diet for 90 days.

CHARACTERIZATION OF TEST AND CONTROL COTTON SEEDS

The test cotton seeds used in this study was cotton hybrid developed using **JKC 738 Bt**. The corresponding control cotton seeds was cotton hybrid developed using the non Bt version of **JKC 738**. The test and control cotton seeds was characterized by the sponsor prior to their use in this study, using either polymerase chain reaction (PCR) or a detection method for the Cry 1Ac protein (ELISA). The characterization data for the test and control substances was achieved with the study records.

Prior to initiation of the study, IVRI conducted the analysis of all samples of the test and control cottonseed for the presence of mycotoxins and pesticides besides compositional analyses of the test and control cottonseeds. The compositional analysis included was proximate principles (protein, fat, ash, carbohydrates, crude fiber), acid detergent fiber, neutral detergent fiber and gossypol. The sponsor will also provide all such data they have generated through certified laboratories for these analyses, if necessary. If results of these analyses' for particular samples demonstrate unacceptable levels of mycotoxins, pesticides, nutrients or toxicants for farm animal feed, these samples were excluded from use in the goat feeding study.

STUDY OVERVIEW

The methods, species of animals and the route of administration described in this protocol are based up on the standard GECD guidelines No. 408 (1993) and DBT guidelines 1998. This procedure deals with handling, maintaining and other procedures to be followed while conducting feeding studies with goats. The test and control cottonseed was mixed in the concentrate mixture. This route of administration was selected because it represents the most likely route of exposure of goat species in their natural habitat. The test and control substances were properly identified as per the detailed specification provided by the Sponsor. Experimental data collection for test and control groups

included were: body weight gain, feed consumption and blood chemistry during the- in-life phase of the experiment and pathological observations at the study termination.

EXPERIMENTAL DESIGN

Goat husbandry is common in rural Indian agriculture. The availability of standard genetically defined goats and dietary and husbandry conditions, also make goats ideal in the Indian context and safety data on this ruminant model is appropriate. The study was conducted as a randomized block design in which 36 goats (18 males and 18 females) were distributed randomly in three treatment groups evenly consisting of a single goat as a replicate. Randomization was done by weight and sex as follows. The 3 heaviest males were randomly allocated, one to each of the 3 treatment groups. Similarly, the next 3 heaviest males were allocated to each of the treatment group and this process will continue until all 18 male goats have been placed in the 3 treatment groups. The same process was followed for randomly placing female goats in each of the treatment groups.

The test was comprised of feeding goats for 90 days regularly with concentrate mixture of which 12.5 % was cottonseed and the concentrate itself was 25% of the total feed i.e. concentrate and green grass. The consumption range of the feed was predetermined. One group of goats (12 goats) was fed with cottonseeds containing the **JKC 738** Bt and the second group (12 goats) was fed with cotton seeds with non **Bt version of JKC 738** & the third group of goats (12 goats) was fed soyabean meal as a substitute for cotton meal. Measurements and observations were made over the duration of the study periodn (90 days).

Animals: Male goats & Female goats

Group I: Soyabean meal

Group II: Cotton Hybrid with non Bt version of **JKC 738**

Group III: Cotton Hybrid with the **JKC 738** Bt trait

All animals in the treatment groups 1 to 3 was fed conventional cotton seeds, i.e. without the **JKC 738 Bt** trait, in their diet during a minimum 30days acclimatization period prior to start of the study. Experimental analysis was initiated during this period itself (feed consumption, weight gain etc). These data were included in the statistical analysis and interpretation of the results.

The study was divided as follows:

1. ACCLIMATIZATION: From receipt of the animals till the initiation of the study (a minimum duration of 30days)

Experimental treatments: 90 days from the end of the acclimatization period.

2. ANIMAL CARE AND FACILITY:

- **Animal Species**

The goat *(Capra hircus)*: The Indian Barberi Breed

- **Source**

Central Institute for Research on Goats, Makhdoom, Mathura, U.P.

- **Health**

All animals were examined by a State certified veterinarian for general health and absence of disease symptoms prior to study initiation.

- **Number of animals**

36 animals (18 males and 18 females): for 90 days feeding

- **Age and weight**

Age of the animals was approximately 12 months and the body weight ranged between 15 and 18 kg.

- **Acclimatization**

The animals were acclimatized for a period of 30 days prior to the actual studies. The goats were given anti-helminthic drugs and also drugs for treatment of ectoparasites before the initiation of the study. The name, manufacturer, dose and date of administration of all drugs was documented. All animals in the treatment groups 1 to 3 were fed conventional cottonseeds in the diet during acclimatization.

- **Animal identification**

Each animal was numbered accordingly with the help of a metal tag around the neck. Their ears (inside) was also marked with permanent marker having the corresponding number to ensure that either of the identification number is available at any given point of time.

- **Housing, environmental conditions and animal care**

Goats were housed individually in well constructed cemented pens and maintained under strict hygienic conditions of veterinary care. Each pen was measuring 2.0 sqm per goat, allowing proper movement to the animals. The floor of the pen was constructed of concrete and the walls of bricks. The roof was made of corrugated sheet. At initiation of the study, each pen was having a single goat and the goat was identifiable by a number. During the test, the temperature in the building was approximately 25-40°C and recorded using a temperature recording chart. When necessary, air cooler was provided to maintain the specified temperature. Relative humidity was recorded at 24 hour intervals. The goats were provided a 16 hour light and 8 hour dark photo periods during the test. Housing and animal husbandry practices were followed as per standard practice for the testing facility.

- **Feed and water**

Clean drinking water was provided *ad libitum*. The feed provided to the animals was analysed once a month according to test laboratory standard operating procedures and a record was maintained of all the analyses obtained during the relevant period of the study. Concentrate mixture was consisted of crushed maize, wheat bran, soyabean meal, crushed cotton seeds (BT & Non-BT), mineral mixture and salt, and daily green oats was fed as a roughage in the diet of goats. The test was comprised of feeding of the goats for 90 days regularly with feed concentrate mixture of which 12.5 % was cottonseed and the concentrate itself was 25% of the total feed i.e. concentrate and green oats. The consumption range of the feed was pre-determined.

- **Bedding**

No bedding was used; instead the floor was made of rough cement/concrete to avoid slipping of goats while walking or standing.

- **Exercise**

Though the goats do not need any strenuous exercise, they were however allowed to go out of their pens in an open field for about 2-3 hours each day but ensuring that they do not take any other foliage. The area of their movement was devoid of any vegetation but water was provided during this period of their routine.

- **Animal diet**

The test and control diets were prepared by blending the test or control substances directly with the concentrate ration in accordance with the Standard Operating Procedure (SOP) available for grinding, mixing/blending of animal diets, while avoiding cross contamination. Blending is normally done with a blender. The method of blending was documented assuring the dietary mixture is homogeneous. Diets were formulated to meet or exceed India's nutrient requirement standards or the NRC Nutrient Requirement Guidelines for Goats. Diet formulation (Table 1) and expected nutrient content was documented (Table 2). The concentrate mixture was prepared every fortnight. The diets were provided to the goats from day 0 to 90 days exposure period. All the feed ingredients were procured in one time that used in the concentrate mixtures were analyzed and data records were maintained. The other ingredients of the concentrate was purchased in bulk and made available for mixing but the mixing and blending of the constituents was done fortnightly. The feed ingredients were stored in a dry and clean room to avoid attack by fungus. The test and control cottonseeds were crushed separately and mixed with the feed concentrate. Samples of mixed concentrate were taken daily for each treatment and stored under refrigeration. After each week, random samples were composited and sub-samples were taken for analysis. The analysis of the feed was for the following parameters: moisture, crude protein, fat, acid detergent fiber, neutral detergent fiber, calcium, phosphorus, magnesium, sodium, potassium, copper, zinc, manganese, iron, vitamin A, vitamin D, vitamin E. The analysis was conducted on the mix and the raw ingredients. Samples of oats were taken daily and dried for further analysis.

MEASUREMENTS AND OBSERVATIONS

All the animals were observed daily for morbidity, mortality and clinical signs.

- **Daily observations**

An in-house veterinarian checked the health of each animal and document that the animal is healthy before assigning the animal to a treatment group. A record was maintained for these remarks by the veterinarian. The general health of all the animals was monitored daily and relevant records were maintained. Any adverse observation was

documented: Animals found moribund or dead during the study period was necropsied to the extent necessary to determine the probable cause.

- **Body weight and temperature**

Body weight was measured weekly at a predetermined time along with their health status. A chart of weekly body temperature recorded was also maintained. The method of measuring body temperature or rectal temperature was documented. Scales were calibrated before the beginning of the study and checked prior to each weighing using weights that encompass the range of what the goats weigh. This calibration was documented.

- **Body weight/feed consumption**

Individual body weight was taken at the initiation of the experiment, every 7 days during the exposure period and at the end of the exposure period. Average feed consumption for individual animal was maintained for the entire period. Determination of feed consumption and body weight was continued, if the study period is extended. Daily feed offered and refused was measured for the concentrate and oats. The amount of concentrate to offer was determined using the average of the previous week's oats consumption. This amount was documented for each goat. If, a goat did not consume target levels of concentrate mixture, the amount of oats offered was restricted to maintain the proportion of oats to concentrate ratio.

- **Feed intake**

Goats were having access to the experimental feed (concentrate mixtures) from 9 a.m. to 12 noon each day.

- **Necropsy and Pathological examinations**

Goats found moribund or dead during the study period were necropsied to the extent necessary to determine the probable reason. Any gross lesions observed at necropsy were processed for histopathological examinations.

- **Hematological observations**

Samples of blood (about 0.5 ml) were drawn from the external jugular vein of individual goat of every group on 0 day, 45 day and 91 day of the study. Various haematological estimations were performed. Following parameters were analyzed:

1. Total RBC count
2. Total WBC Count
3. Differential leucocytic count
4. Haemoglobin concentration

BLOOD EXAMINATION

For examination of the collected blood for differential leukocyte count (DLC), Total Leukocyte Count (TLC) and Total Erythrocyte Count (TEC) examination the procedure of Jain (1986) was adopted. Hemoglobin concentration was estimated by cyanmethemoglobin method and expressed in g/dl.

BIOCHEMICAL EXAMINATION

COLLECTION OF SERUM SAMPLES

From jugular vein about 10 ml blood was collected in a sterilized test tube without any anticoagulant at 15 days interval. Tubes containing blood were kept at room temperature. Clot appeared within 2 hours and that was broken with the help of Pasteur pipette within one hour. Serum was collected with the help of a micropipette. Centrifugation of serum was performed and it was stored in refrigerator at -4^0C in labeled glass vials.

Following parameters were estimated

Total serum protein. It was determined spectrophotometerically by the biuret method

Glucose: It was determined spectrophotometerically by GOD/POD method

Blood urea nitrogen: It was determined spectrophotometerically by DAM method

Bilirubin It was determined spectrophotometerically by Malloy and Evelyn method

GOT: It was determined spectrophotometerically by DNPH method

GPT: It was determined spectrophotometerically by DNPH method

Alkaline phosphatase It was determined spectrophotometerically by Kind and Kings method

LDH It was determined spectrophotometerically by DGKC UV method.

DIGESTION OF FEED SAMPLES

The feed samples were digested by the method of Trolson (1969). One gram of previously ground and stored samples was taken in digestion tube and 5ml of concentrated HNO_3 and 1 ml of concentrated H_2SO_4 were added and mixed well. The samples were kept overnight at room temperature followed by digestion on low heat (70-80^{0}C) using heat block (digestion bench), until the volume of samples reduced to about 1 ml. To this 3 ml of double acid mixture (3 part concentrated HNO_3 and 1 part 70% $HClO_4$) was added and low heat digestion continued until the white fumes appeared from the samples. Digested samples were diluted with 2ml triple distilled water and filtered through Whatman filter paper No. 1. Taking 0.5ml triple distilled water did repeated washing of digestion tube and filter paper. The filtrate was again diluted with triple distilled water to make the final volume to 10 ml.

DIGESTION OF SERUM SAMPLES

Serum samples were digested as per procedure described by Kolmer *et. al.*, (1951). To 3 ml of serum equal volume of concentrated HNO_3 was mixed in the digestion tube. The samples were kept overnight at room temperature followed by digestion on low heat (70-80^{0}C) using heat block (digestion bench), until the volume of samples reduced to about 1 ml. To this 3 ml of double acid mixture (3 part concentrated HNO_3 and 1 part 70% $HClO_4$) was added and low heat digestion continued until the digested samples became watery clear and emitted white fumes. As per need, the addition of 3 ml double acid mixture followed by low heat digestion was repeated couple of times. Further, heating was continued to reduce the volume to approximately 0.5ml. Final volume of 10 ml was made with triple distilled water after warming the solution.

ESTIMATION OF PHOSPHORUS FROM FODDER

Phosphorus in fodder was estimated by making an acid extract of the ashed material. A suitable quantity of the sample (3 to 5g) was taken in a previously weighed silica crucible. The sample was charred to make it smoke free on an electric heater. The silica crucible with its contents was kept in a muffle furnace at about 550^{0}C for 3 hrs. The crucible with completely ashed sample was taken out and kept in a desiccator to cool. The weight of the ash with crucible was again recorded. The ash was transferred to a

beaker quantitatively with water and 20-25 ml of hydrochloric acid was added to the beaker. The content of the beaker was boiled carefully to avoid spurting for 15 minutes. The acid extract was filtered through Whatman filter paper No. 1 into a 250 ml standard volumetric flask quantitatively by repeated washings with hot distilled water. This mineral extract was used for the estimation of phosphorus.

Phosphorus was estimated by the method of Talapatra *et. al.*, (1940). A known quantity of aliquot of the acid extract was taken in a 250 ml beaker. Phosphorus was precipitated as ammonium phosphomolybdate by adding nitric acid-ammonium molybdate solution (equal volume of nitric acid and 20 per cent ammonium molybdate solution). The precipitate was left overnight and the supernatant was filtered through Whatman filter No. 42. Precipitate was washed first with 2% nitric acid, thereafter with 3% potassium nitrate solution till no acid reaction was shown by the filtrate. The precipitate was dissolved by excess volume of N/7 sodium hydroxide solution. The excess alkali was back titrated with N/7 nitric acid solution using phenolphthalein as indicator. Thus, the exact volume of N/7 sodium hydroxide required to dissolve the precipitate was obtained. The phosphorus content of the sample was calculated by the formula: -

1 ml of N/7 sodium hydroxide = 0.0001925 g of phosphorus

$$\text{Percent phosphorus} = \frac{\text{ml of N/7 NaOH} \times 0.0001925 \times \text{aliquot factor} \times 100}{\text{Weight of the sample on dry matter basis}}$$

MACRO AND MICRO MINERALS ANALYSIS

The content of calcium, magnesium, potassium, sodium, zinc, copper, cobalt, selenium and iron in soil, fodder and serum samples were estimated by using Atomic Absorption spectrophotometer (AAS-4141 ECIL, India) after digesting the samples.

ESTIMATION OF SERUM INORGANIC PHOSPHORUS

The serum inorganic phosphorus was estimated by the method of Taussky and Shorr (1953). Phosphorus in the form of inorganic phosphate was allowed to react with molybdic acid, producing the phosphomolybdate complex. This was reduced to a blue-coloured compound that is proportional to the phosphorus concentration.

NECROPSY

All the animals were sacrificed at the end of study based on the Test Facility SOP. Goats were sacrificed by administration of a saturated solution of magnesium sulphate intravenously and autopsy carried out by the veterinary pathologist of the study following standard procedure. Also the carcass was disposed off following the SOP available for the said purpose and documented.

- **Organ weights**

  The gross lesions in the organ were noted and weights of the following organs were recorded:

  1. Adrenals,
  2. Heart,
  3. Liver,
  4. Gonads (testes-and ovaries), Brain and Kidneys,
  5. Spleen

Histopathological examinations

Following organs were preserved in 10% buffered formalin:

Adrenals, Kidneys, Testes, Liver, Thymus, Lungs, Colon, Spleen, Ovaries, Stomach (all 4 compartments), Heart, Small intestine.

Histopathological examinations of these organs were conducted, by a qualified pathologist, only if gross lesions are noted.

The tissues were subjected to dehydration procedures and processed in a tissue processor through different grades of alcohol and cleared in chloroform. The tissues were embedded in paraffin wax, sectioned at 7 to 10 microns and stained with Haematoxylin-Eosin.

DISPOSAL

The carcasses were burnt using incinerator/furnace.

RESULTS

The experiment was conducted on thirty six Barberi goats, (eighteen male and eighteen female) aged 12 months average body weight 17.97±0.37, divided into three equal groups containing 6 male and 6 female in each following randomized block design. Animals were de-wormed, vaccinated and acclimatized to the new environment before the start of the experiment. All the goats were housed individually in a well constructed, cemented pens and maintained under strict hygienic conditions. Animals were maintained as per the following schedule.

Attributes	**Control Group**	**Experimental Non-Bt**	**Experimental Bt**
Concentrate	Control Conc. Mix.	Expt. Conc. Mix. (Non-Bt)	Expt. Conc. Mix. (Bt)
Roughage	Oats *ad libitum*	Oats *ad libitum*	Oats *ad libitum*
Feeding Time	10.30 AM	10.30 AM	10.30 AM
Body weight	Fortnightly	Fortnightly	Fortnightly
Temp. recording	Weekly	Weekly	Weekly

All the goats were fed concentrate mixtures (Table 1 & 2) @ 250 g each and green oats/ hay throughout the experimental period. Animals were provided clean and fresh drinking water *ad libitum* daily. Recording of body weight was done at fortnightly interval. Body temperature was recorded at weekly interval. However, dry matter intake was recorded daily and presented on weekly basis. From the data recorded to date it is

evident that there was no difference in all the three groups with respect to body weight change (Table 3), body temperature (Table 4) and daily dry matter intake (Table 5).

Table 1. Ingredients composition of concentrate mixtures

Ingredients	Concentrate mixture Control	Conc. mixture Expt. Non-Bt	Conc. mixture Expt. Bt
Maize	40	40	40
Wheat bran	32	30	30
Soyameal	25	14.5	14.5
Cotton seeds (Non-Bt)	--	12.5	--
Cotton seeds (Bt)	--	--	12.5
Mineral mixture	2	2	2
Common salt	1	1	1

Table 2. Mean chemical composition (% on DM basis) of different feed ingredients and concentrate mixtures

Feed ingredients & concentrate mixtures	DM*	OM	N	CP	EE	NDF	ADF	Ash	AIA
Wheat bran	92.16	95.07	2.69	16.86	6.63	44.55	10.53	4.92	0.30
Maize	91.48	97.35	2.07	12.96	3.43	32.51	4.23	2.65	0.47
Soybean meal	92.08	91.63	8.37	52.31	1.53	13.29	10.41	8.37	1.62
Oats	36.70	86.24	1.52	9.53	3.96	64.98	42.20	13.76	8.39
Cotton seeds (Non-Bt)	94.50	94.99	5.26	32.88	31.20	20.05	13.22	5.01	0.22
Cotton seeds (Bt)	94.02	94.98	4.58	28.66	26.88	23.57	16.29	5.02	0.22
Conc. mixture Control	93.19	90.65	3.85	24.07	3.05	24.11	8.47	9.35	1.11
Conc mixture (Non-Bt)	92.28	93.12	3.99	24.94	6.46	28.17	9.35	6.88	0.62
Conc. mixture (Bt.)	92.79	93.09	3.90	24.72	4.45	21.80	9.51	6.91	0.83
Oat Residue-I	59.76	88.59	1.50	9.30	2.11	76.63	50.92	11.41	4.28
Oat Residue-II	57.17	86.77	1.45	9.06	2.57	73.51	47.44	13.23	5.95
Oat Residue-III	55.68	87.29	1.48	9.20	1.85	74.73	53.59	12.71	5.49

*Content on % fresh basis; DM-dry matter, OM-organic matter, N-nitrogen, CP-crude protein, NDF-neutral detergent fibre, ADF-acid detergent fibre and AIA-acid insoluble ash

Table 3. Body weight changes of goats in different groups

Fortnight	Control Group	Experimental Non-Bt	Experimental Bt
0	14.9±0.45	15.1±0.80	15.6±0.90
1	15.0±0.56	16.4±0.97	16.9±1.24
2	17.7±1.11	17.3±0.84	18.6±1.28
3	17.7±1.04	17.5±0.84	18.6±1.25
4	17.2±0.94	17.1±0.89	18.0±1.18
5	17.7±1.05	17.0±0.82	18.3±1.14
6	19.7±1.11	18.5±0.94	19.4±1.28

Table 4. Weekly temperature record of animals in different groups

Weeks	Control Group	Experimental Non-Bt	Experimental Bt
0	103. 36±0.20	103.32±0.17	103.45±0.18
1	103.53±0.32	103.95±0.35	103.03±0.35
2	103.47±0.26	103.78±0.27	103.45±0.38
3	103.45±0.34	103.49±0.27	103.65±0.33
4	103.57±0.21	103.12±0.34	103.85±0.17
5	103.87±0.33	103.03±0.20	103.63±0.17
6	103.03±0.19	104.00±0.18	104.13±0.23
7	103.52±0.16	103.62±0.21	104.92±0.13
8	103.00±0.23	103.85±0.27	103.90±0.15
9	103.73±0.18	103.47±0.17	103.00±0.14
10	103.71±0.17	103.55±0.17	103.95±0.13
11	103.96±0.42	104.22±0.24	103.87±0.20
12	103.3±0.10	104.2±0.07	104.6±0.12
13	103.6±0.14	104.6±0.11	104.0±0.13

Table 5. Dry matter intake (g/d) of animals in different groups

Weeks	Control Group	Experimental Non-Bt	Experimental Bt
0	387.5±8.57	415.4±15.63	392.0±23.30
1	419.7±11.46	411.4±17.79	415.5±20.10
2	393.0±17.31	401.6±14.86	423.5±15.90
3	389.6±13.75	381.9±12.80	408.7±17.96
4	485.9±13.76	486.2±16.18	506.23±17.77
5	542.4±24.11	497.0±20.20	543.31±34.29
6	541.7±20.14	529.6±17.49	553.73±19.91
7	559.3±14.22	561.1±18.56	583.36±19.36
8	512.9±20.30	454.3±19.12	517.0±16.54
9	453.7±17.34	463.3±14.55	470.3±15.62
10	436.8±15.15	432.8±16.22	463.1±17.81
11	507.8±22.73	489.8±20.90	500.2±18.47
12	636.5±26.61	574.0±25.57	626.0±30.25
13	660.6±35.18	648.4±31.29	667.5±31.45

NECROSCOPIC FINDINGS

OBSERVATIONS ON BIOMETRY

1. Absolute and relative weight (RW) of brain, heart, lungs, liver, spleen, adrenals, kidneys, gonads (ovaries/testes) were recorded separately in different groups of male and female goats sacrificed on 90 DPF (Table 6).
2. In BT Cotton fed group only in females, relative weight of heart was decreased while liver increased as compared to Controls.
3. In Indian Cotton fed group relative weight of lungs and spleen of females was decreased while weight of liver was increased, whereas in males only relative weight of liver was decreased as compared to Controls.

These studies indicated that BT Cotton had insignificant effect on organ biometry as compared to Indian Cotton and Control groups.

On postmortem examination none of the goat belonging to BT Cotton or Indian Cotton groups showed lesions of toxicity.

However, goats of all three groups showed incidental lesions like enlarged and oedematous mesenteric lymph nodes, abscesses in bronchial/inguinal lymph nodes, presence of *Haemonchus contortus* worms in abomasum, cysts of *Cysticercus tenuicollis* or hydatid in some of visceral organs. Certain other minor lesions like subcutaneous/pulmonary abscesses, atrophied testes/adrenals were seen. These types of lesions are not uncommon in Indian goats and not induced by feeding of BT Cotton.

Table: 6 Relative organ weight of BT Cotton fed goats sacrificed at 90 days post feeding.

Anim. No.	Body weight	Group/ Sex/n	Organs								
			Brain	*Heart*	*Lungs*	*Liver*	*Spleen*	*Adrenals*	*Kidneys*	*Ovaries*	*Testes*
	Group-I	*Control*									
13	16.0	F	0.52	0.58	1.27	1.65	0.26	0.01	0.35	0.01	-
23	17.0	F	0.46	0.53	1.66	1.93	0.23	0.01	0.35	0.01	-
Mean	**16.5**	**n(2)**	**0.49**	**0.55**	**1.46**	**1.79**	**0.24**	**0.01**	**0.35**	**0.01**	
28	21.0	M	0.36	0.40	1.18	1.81	0.24	0.01	0.30	-	0.81
42	24.4	M	0.34	0.34	1.53	1.84	0.22	0.01	0.29	-	0.61
Mean	**22.7**	**n(2)**	**0.35**	**0.37**	**1.36**	**1.83**	**0.23**	**0.01**	**0.30**	**-**	**0.71**
	Group-II	*Ind.Cott.*									
21	16.6	F	0.52	0.41	1.00	1.99	0.17	0.01	0.31	0.01	-
24	14.2	F	0.55	0.37	1.44	2.09	0.17	0.01	0.37	0.01	-
Mean	**15.4**	**n(2)**	**0.53**	**0.39**	**1.22**	**2.04**	**0.17**	**0.01**	**0.34**	**0.01**	**-**
29	16.6	M	0.38	0.40	1.23	1.61	0.35	0.01	0.23	-	0.81
38	14.2	M	0.42	0.40	1.43	1.86	0.19	0.01	0.29	-	0.84
Mean	**15.4**	**n(2)**	**0.40**	**0.40**	**1.33**	**1.73**	**0.27**	**0.01**	**0.26**	**-**	**0.82**
	Group-III	*BTCott.*									
10	15.0	F	0.47	0.46	1.45	2.03	0.31	0.01	0.33	0.01	-
17	16.8	F	0.55	0.41	1.51	1.92	0.24	0.01	0.35	0.01	-
Mean	**15.9**	**n(2)**	**0.51**	**0.43**	**1.48**	**1.97**	**0.28**	**0.01**	**0.34**	**0.01**	**-**
43	24.0	M	0.40	0.35	1.40	1.62	0.23	0.01	0.28	-	0.59
44	21.0	M	0.40	0.38	1.32	1.97	0.21	0.01	0.31	-	0.82
Mean	**22.5**	**n(2)**	**0.40**	**0.36**	**1.36**	**1.79**	**0.22**	**0.01**	**0.29**	**-**	**0.70**

HAEMATOLOGICAL STUDIES

Haemoglobin

Table –7 reveals that there was no significant change in the haemoglobin levels in the Bt cotton seed fed goat as compared to the non Bt cotton fed goat and the control group. The values of Haemoglobin in the Bt cotton seed fed goats was in normal range during the entire study period. This trend continued during the entire experimental period of 90 days.

Total RBC

In case of RBC the values of the control group, Non Bt cotton seed fed group and Bt cotton seed fed group were similar. There was no significant change in the values of RBC during various days of experimental studies. These values remained within the normal range during the entire experimental study of 90 days (Table-7).

White blood Cells (WBC)

In case of WBC the values of the control group, Non Bt cotton seed fed group and Bt cotton seed fed group were almost similar in all the groups. There was no significant change in the values of WBC during various days of experimental studies. These values remained within the normal range during the entire experimental study of 90 days (Table-7).

Differential Leucocytes Count (DLC)

Regarding the Differential leukocyte counts (DLC) it was observed from the perusal of Table 7 that the values of Neutrophils, Lymphocytes, Eosinophils, Basophils, Monocytes were in the normal range in group fed with Bt cotton seed when compared to the Normal control group. This trend continued throughout the experimental study of 90 day and the values of Neutrophils, Lymphocytes, Eosinophils, Basophils, Monocytes remained in normal range in all the three groups viz control group, Non Bt cotton seed fed group and Bt cotton seed fed group.

Table 7 Showing haematological status of the Bt Cotton fed and non-fed groups of goats during various days of treatment

Days/ Groups		Haemoglobin (g/dl)	RBC (millions/cmm)	WBC (thousands/cmm)	Neutrophils	Lymphocytes	Eosinophils	Basophils	Monocytes
0 Day	Control	10.84	11.92	6.01	44	52	2	0	2
	T1	10.68	11.98	5.94	43	53	2	1	1
	T2	10.72	11.92	5.94	42	54	1	1	2
45 day	Control	10.66	11.96	5.89	41	54	2	1	2
	T1	10.78	11.95	6.12	43	54	2	0	1
	T2	10.66	11.98	6.02	42	53	1	2	2
90 day	Control	10.76	11.94	6.22	42	53	2	1	2
	T1	10.68	11.93	6.10	38	58	2	1	1
	T2	10.84	11.96	5.98	40	56	1	1	2

SERUM BIOCHEMICAL STUDIES

Total Proteins

Table 8 shows that the values of total protein in all the three groups were similar and within the normal range through out the experimental period of 90 days. The values were in normal range of 5.9g/dl to 6.7 g/dl. No variation was observed in the goat fed with the Bt cotton seed during the entire experimental study of 90 days and the values corresponded to the values of the 0 day of study.

Serum glucose

In case of serum glucose, there were no significant alterations in the values in the three groups of animals viz control group, Non Bt cotton seed fed group and Bt cotton seed fed group (Table 8). The serum glucose values showed normal trend and were within the normal range of healthy animals during the entire experimental period.

Blood urea nitrogen

Blood urea nitrogen values of the control group, Non Bt cotton seed fed group and Bt cotton seed fed group were within normal range at the 0 day of the experimentation (Table-8). There were no significant changes in the values of BUN during the entire experimental period and its value remained within normal range during 15^{th}. 30^{th}, 45^{th}, 60^{th}, 75^{th} and 90^{th} day of experimentation.

Serum bilirubin

Table 8 reveals that serum bilirubin values were in normal range i.e. 0.3 to 0.6 mmol/l in group fed with Bt cotton seed when compared to the normal control group. During the entire period of study the values of all the three groups remained in normal range and there was no significant alteration in these values in respect of serum bilirubin.

SGOT

The values of SGOT were in normal range in all the goats of three groups and these trends were observed during the entire period of experimental study. There was no variation in the Bt cotton seed fed group when compared to the normal healthy groups.

SGPT

Similar to the SOT there was no significant effect of treatments on the values of SGPT, which remained within the normal range in the three groups.

Alkaline phosphate

In case of alkaline phosphate the values were showing normal trend as evident from the table 8 in all the groups viz. control group, Non Bt cotton seed fed group and Bt cotton seed fed group through out the study.

LDH

Table 8 reveals that the values of serum LDH were within the normal range in all the three groups of animals and these values remained in normalcy during the entire experimental study in animal of all the three groups.

Table-8 showing Serum biochemical status of the Bt Cotton fed and non Bt cotton-fed groups of goats during various days of treatment

Days/ Groups		Total serum protein (g/dl)	Glucose (mg./dl)	Blood urea nitrogen (mmol/l)	Bilirubin mmol/l)	GOT (iu/l)	GPT (iu/l)	Alkaline phosphatase (iu/l)	LDH (14/l)
0 Day	Control	5.9	56	3.8	0.4	161	33	68	140
	T1	6.0	54	4.7	0.4	168	28	78	130
	T2	6.2	56	4.8	0.3	174	34	74	128
15 day	Control	5.9	58	4.5	0.5	357	36	166	126
	T1	6.2	61	4.9	0.6	364	38	168	125
	T2	6.4	57	4.8	0.4	350	41	171	123
30 Day	Control	6.0	67	3.7	0.3	207	48	190	160
	T1	6.2	74	4.5	0.4	228	38	160	145
	T2	6.4	59	4.7	0.5	218	42	154	134
45 day	Control	6.4	66	6.5	0.5	355	45	190	186
	T1	6.2	64	5.1	0.6	310	68	150	190
	T2	6.5	59	4.9	0.4	365	56	174	192
60 day	Control	6.2	67	4.8	0.4	356	53	185	264
	T1	6.7	62	5.4	0.5	335	68	208	311
	T2	6.4	63	4.9	0.6	330	71	156	268
75 day	Control	5.9	67	6.4	0.5	398	65	296	356
	T1	6.6	62	6.2	0.6	405	70	269	360
	T2	6.3	68	6	0.4	412	72	288	345
90 day	Control	6.2	67	4.8	0.4	356	53	185	264
	T1	6.7	62	5.4	0.5	335	68	208	311
	T2	6.4	63	4.9	0.6	330	71	156	268

SERUM MINERAL STATUS

Macro Minerals

Table 9 shows that Ca, P and Mg values were in the normal range in group fed with Bt cotton seed when compared to the normal control group. This trend continued through out the experimental study of 90 day and the values of Ca, P and Mg remained in normal range in all the three groups viz. control group, Non Bt cotton seed fed group and Bt cotton seed fed group.

Regarding serum Na and K values there were no significant changes as observed during entire experimental study of 90 days. The values remained within the normal range on various days of experimental studies.

Micro Minerals

Similarly serum Cu and Zn values were in normal range in all the goats of three groups and these trends were observed in the entire period of experimental study. The serum Cu values were in range of 0.61ppm to 0.69 ppm and that of serum Zn were in range of 3.8ppm to 4.20 ppm in all the three experimental groups.

From the perusal of table 9 there was no significant change in the serum Mn and Fe values in the Bt cottonseed fed goats as compared to the non Bt cotton fed goats and the control group showing normal trend through out the experimental study of 90 days. The values of serum Fe was within normal to higher side in all the samples.

Table –9 showing Serum mineral status of the Bt Cotton fed and non fed groups of goats during various days of treatment

Days/ Groups		Ca (mg/dl)	P (mg/dl)	Mg (mg/dl)	Na (ppm)	K (ppm)	Cu (ppm)	Zn (ppm)	Mn (ppm)	Fe (ppm)
0 Day	Control	8.8	4.0	2.6	139	5.3	0.62	4.12	0.71	14
	T1	8.2	4.1	2.7	148	5.2	0.64	4.14	0.72	15
	T2	8.4	3.8	2.6	145	4.8	0.66	4.15	0.71	14
15 day	Control	8.6	4	2.6	131	5	0.63	3.8	0.72	14
	T1	8.5	3.6	2.6	136	4.5	0.61	4.06	0.71	16
	T2	9.2	3	2.5	137	4.3	0.64	4.10	0.70	11
30 Day	Control	8.5	4.2	2.6	135	5.3	0.65	4.04	0.71	16
	T1	8.4	4.5	2.9	138	5.1	0.62	4.02	0.73	12
	T2	8.6	3.8	2.8	140	4.2	0.61	4.05	0.76	15
45 day	Control	8.8	4.1	2.8	141	5.2	0.64	4.02	0.74	12
	T1	8.8	4.4	2.6	144	5.6	0.65	4.18	0.72	11
	T2	8.4	4.2	2.7	146	4.4	0.61	4.09	0.71	18
60 day	Control	10.1	4.8	2.8	144	5.8	0.68	4.11	0.75	14
	T1	9.8	4.7	2.7	137	5.6	0.65	4.12	0.72	15
	T2	9.2	4.4	2.5	148	4.8	0.63	4.18	0.74	19
75 day	Control	8.8	4.2	2.64	142	5.2	0.62	4.08	0.78	13
	T1	9.2	4.6	2.72	139	5.1	0.64	4.16	0.71	14
	T2	9.4	4.8	2.65	144	4.5	0.61	4.20	0.73	10
90 day	Control	10.4	4.4	2.6	145	5.7	0.69	4.13	0.76	16
	T1	9.6	4.5	2.5	139	5.2	0.62	4.15	0.78	18
	T2	9.2	4.2	2.4	147	4.7	0.61	4.19	0.75	17

COMPOSITE FEED ANALYSIS

Fodder/Feed provided to the experimental animals (goats) was analysed for various mineral content viz: Calcium (Ca), Phosphorus (P), Magnesium (Mg), Potassium (K) Sodium (Na) Iron (Fe), Copper (Cu), Zinc (Zn) and Manganese (Mn). These samples were analysed randomly on 0, 30,60 and 90 day of the experimental study.

On analysis of these samples the values of these minerals were found to be within the normal range during the various days of analysis of feed provided to the experimental animals. It can be safely concluded that feed fed to the experiment animals was rich in all the inorganic components and no deficiency of these minerals was observed during the entire experimental study (Table-10).

Table 10 showing the mineral status of the composite feed fed to goats during study

Attribute/Day	**0 day**	**30th day**	**60th Day**	**90th day**
Ca%	0.42	0.44	0.42	0.43
P%	0.32	0.36	0.32	0.35
Mg%	1.00	0.9	1.08	1.14
Na%	0.07	0.05	0.08	0.06
K%	0.75	0.72	0.71	0.76
Fe%	0.05	0.08	0.06	0.04
Cu (ppm)	13	12	14	11
Zn (ppm)	15	16	14	18
Mn (ppm)	115	111	117	109

Vitamin contents in feeds, concentrate mixtures and composite diets (approximate values)

Vitamin contents in feeds, concentrate mixtures and oat fodder are presented in table 11. The values of vitamin A (converted form carotene @ 450 i.u. per beta carotene for goat) and Vitamin E only were detectable; and for tropical countries like India practically there is no need to bother about Vitamin D availability due to adequate exposure of sunlight through out the year.

Table 11. Vitamin A and E in feeds, concentrate mixtures and fodders

Feeds	**Vitamin A activity (IU)**	**Vitamin D**	**Vitamin E (IU)**
Maize (per Kg)	437	Non-detectable	23.3
Wheat bran (per Kg)	____	Non-detectable	21.7
Concentrate Mixtures (Per Kg)			
Control group	4175	80	16.26
Non-BT cotton group	4172	79	15.83
Bt- cotton group	4172	79	15.83
Fodder (per Kg)			
Green oats	127,350	80	143.3
Oats hay	126,000	traces	141.7
Approximate estimate of vitamins intake/day			
Control group			
Conc. Mix (200g)	835	16	3.25
Fodders (300g)	38000	Traces	42.75
Total intake	38835	16+	46.00
Non-Bt group			
Conc. Mix.	835	16	3.17
Fodder	38000	Traces	42.75
Total intake	38835	16+	45.92
Bt- group			
Conc. Mix.	835	16	3.17
Fodder	38000	Traces	42.75
Total intake	38835	16+	45.92

From the table 11 it is quite clear that there was no difference in the values of vitamin A activity and vitamin E contents of non-Bt cottonseed and Bt-cotton seed. Oats fodder was highly rich in vitamin E content due to which experimental goat received adequate supply of these vitamins. The values of vitamin D in concentrate mixtures and approximate feed intake are the expected availability from added vitamin D_3 in the concentrate mixtures.

Summary

To assess sub-chronic oral toxicity of feeding genetically engineered cotton seeds developed by JK Agri Genetics Ltd in goats, an experiment of 90 days duration was planned on thirty six Barbari goats (eighteen male and eighteen female) aged 12 months average body weight 17.97±0.37 divided into three equal groups containing 6 males and 6 females in each following randomized block design. Animals were de-wormed, vaccinated and acclimatized to the new environment before the start of the experiment. All the goats were housed and fed individually in a well constructed, cemented pens and maintained under strict hygienic conditions.

All the goats were fed concentrate mixtures @ 250 g each and *ad libitum* green oats/ hay throughout the experimental period. Animals were provided clean and fresh drinking water *ad libitum* daily. Recording of body weight was done at fortnightly interval. Body temperature was recorded at weekly interval. However, dry matter intake was recorded daily and presented on weekly basis. Initial body weight of animals were 14.9±0.45, 16.1±0.80, 15.6±0.90 kg in group 1, 2 and 3, respectively. There was no significant difference in body weight change in all the three groups (Table 3). Body temperature of the goats in all the groups was similar and remained within the normal range (Table 4). Daily dry matter intake (g) was also similar in all the three groups, which varied from 387.5±8.57 to 660.6±35.18 in group 1, 381.9±12.8 to 648.4±31.29 in group 2 and 392.0±23.30 to 667.5±31.45 in group 3 (Table 5). From the data recorded to date it is evident that there was no difference in all the three groups with respect to body weight change, body temperature and daily dry matter intake.

These studies indicated that BT Cotton had ~~insignificant effect~~ no statistically significant effect on organ biometry as compared to Indian Cotton and Control groups. On postmortem examination none of the goat belonging to BT Cotton (JKC) or Indian Cotton groups showed lesions of toxicity. However, goats of all three groups (including the control group) showed incidental lesions like enlarged and oedematous mesenteric lymph nodes, abscesses in bronchial/inguinal lymph nodes, presence of *Haemonchus contortus* worms in abomasum, cysts of *Cysticercus tenuicollis* or hydatid in some of visceral organs. Certain other minor lesions like subcutaneous/pulmonary abscesses, atrophied testes/adrenals were seen. These types of lesions are not uncommon in Indian goats and not induced by feeding of BT Cotton (Table-6).

The results obtained from the experiment showed no evidence of any harmful effect of feeding diets containing Bt cotton on goats. The animals in all groups exhibited similar behaviour and were found normal during the entire experimental period. There was no variation in the haematological (haemoglobin, WBC and DLC), biochemical (Total serum protein, Glucose, Blood urea nitrogen, Bilirubin, GOT, GPT, Alkaline phosphatase, LDH) and serum mineral status (Calcium (Ca), Phosphorus (P), Magnesium (Mg), Potassium (K) Sodium (Na) Iron (Fe), Copper (Cu), Zinc (Zn) and Manganese (Mn)). It can be concluded that there was no toxicity of any kind in the goats fed with Bt Cotton seeds. Further the various haematological and biochemical values were within the normal range and almost similar to the goats fed with non Bt.Cotton Seeds and normal healthy goats of control group.

Certificate of acceptance of the final report of the project report

SUBCHRONIC ORAL TOXICITY IN GOATS – 90 DAYS STUDY FORGENETICALLY ENGINEERED SEEDS.

We, Dr. M.P. Yadav, Director Indian Veterinary Research Institute, Izatnagar 243122 and Dr. P.S. Bhattacharya, Dy. General Manager (Biotech) JK Agri Genetics Ltd.,1-10-177 , 4th floor Varun Towers, Begumpet Hyderabad-500016 hereby certify that the Final report of the project entitled SUBCHRONIC ORAL TOXICITY IN GOATS – 90 DAYS STUDY FOR GENETICALLY ENGINEERED SEEDS was undertaken by Indian Veterinary Research Institute, Izatnagar 243122 as sponsored project has been duly approved and accepted by both the organizations. It is agreed by both the parties that they will not accept any comments or suggestions that may arise in future on account and review of the report by either of us or a third party.

Date. 23/7/2005

Place : **IVRI, Izatnagar (U.P.)**

Name : **Dr. M.P. Yadav**

Designation : **Director, IVRI, Izatnagar**

ICAR Institute

Bhattacharya 23/07/05

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Sponsoring Organisation/ Contracting party