PROJECT NO. : TOX-346G
PRODUCT : Bt COTTONSEEDS
STUDY : ALLERGENICITY STUDY
REPORT NO. : 000046377
DATE : 14.05.2007

ALLERGENICITY STUDY

With

Bt COTTONSEEDS

Report for:

METAHELIX LIFE SCIENCES PRIVATE LIMITED
PLOT NO.3, KIADB 4th PHASE, BOMMASANDRA,
BANGALORE-560 099, INDIA

Guidelines:

'DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts'

Prepared by:

DEPARTMENT OF TOXICOLOGY
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QUALITY ASSURANCE STATEMENT

This is to certify that the work described in the study report entitled 'Allergenicity study' with 'Bt Cottonseeds' has been audited and examined with respect to the study protocol and the Standard Operating Procedures in accordance to 'DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' in compliance with Good Laboratory Practices (G.L.P.) for non clinical laboratory studies.

The report provides true and accurate record of results obtained.

The dates of inspections and dates on which findings were reported to the study director & SRI management are given below:

Phases of the study	Date of inspection	Date of reporting
Protocol	13.12.2006	13.12.2006
Conduct	16.12.2006	16.12.2006
	20.02.2007	20.02.2007
Record and raw data	23.02.2007	23.02.2007
Report	13.05.2007	13.05.2007

Sr. SCIENTIST
QUALITY ASSURANCE

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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

We, the undersigned take overall responsibility to conduct the work described in the study entitled 'Allergenicity study' with 'Bt Cottonseeds' performed with respect to the study protocol and the Standard Operating Procedures in accordance to 'DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts' for non-clinical laboratory studies.

All the raw data, documentation, protocol and copy of final report are retained in the archives at Shriram Institute for Industrial Research, Delhi.

STUDY DIRECTOR **SCIENTIST PATHOLOGY** **HEAD, DEPT. OF TOXICOLOGY**

Approved for issue

DEPUTY DIRECTOR (MANAGEMENT)

SCIENTIFIC PERSONNEL INVOLVED IN THE STUDY

Dr. Vivek Srivastava
(Research Associate)

Mr. Manoj Kumar
(Sr. Analyst)

Dr. Rajendra Palkhade
(Project Associate)

Ms. Arpita Jaiswal
(Analyst)

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SUMMARY

This study was carried out to determine the allergic reactions induced by test substance 'Bt Cottonseeds' with reference to 'Non Bt Cottonseeds' in rabbits. The following battery of tests were conducted to determine the allergic potential of the 'Bt Cottonseeds'.

1. Passive Cutaneous Anaphylaxis test (PCA)
2. Prausnitz-Kustner test (PK)
3. ELISA test

1. The Passive cutaneous Anaphylaxis test (PCA) involves the observation of well defined blue areas indicating the sites of antigen-induced extravasation of fluid due to interaction with tissue fixed antibody when 0.1 ml of test substance is injected intradermally. After 24 hours, 0.6 ml of test substance was injected intravenously together with 0.4 ml of Evan's blue.

2. The Prausnitz-Kustner (PK) test involves the measurement of diameter and intensity of the developed skin lesions, when 0.05 ml of the test substance injected intradermally followed by challenge with 0.05 ml of the test substance intradermally after 24 hours.

3. Enzyme linked immunosorbant assay (ELISA) detect the levels of IgE (if present in the test serum) and solid phase anti-IgE, which can be measured spectrophotometrically.

On the basis of the Passive cutaneous anaphylaxis (PCA) test, Prausnitz-Kustner (PK) test and ELISA, the test substance 'Bt Cottonseeds' was found to be non-allergenic.

INTRODUCTION

- This study was carried out to determine the allergic reactions induced by the test substance 'Bt Cottonseeds' in rabbits. The test substance was administered to the test animals by oral route in dietary preparation.

- This study is designed to evaluate the allergenic potential of test substance 'Bt Cottonseeds'.

OBJECTIVES

(a) To determine the allergic potential of the test substance when administered orally (dietary).

(b) To obtain information on allergic reactions likely to arise after repeated exposure.

TEST SUBSTANCE

The sponsor is responsible for necessary characterization and evaluation of the test substance. The details of the test substance provided by the sponsor are as follows:

PRODUCT NAME	:	NON-Bt COTTONSEEDS (SAMPLE I) & Bt COTTONSEEDS (SAMPLE II)
SPONSOR	:	METAHELIX LIFE SCIENCES PRIVATE LIMITED
MATERIAL DESCRIPTION	:	YELLOWISH BROWN COLOURED POWDER
PACKED IN	:	BROWN COLOURED PAPER CARTONS
DATE OF COMMENCEMENT OF STUDY	:	16. 12. 2006
DATE OF COMPLETION OF STUDY	:	28.03. 2007

Note: For characterization details of test samples, see Annexure – I provided by the sponsor.

EXPERIMENTAL DESIGN

Name of species	:	Rabbits
Strain of the animals	:	New Zealand white
No. of animals used per group	:	10
Age of the animals used	:	6 to 8 weeks
Weight range	:	1.5- 2.0 Kg
Acclimatization period	:	7 Days
Vehicle	:	Conventional diet

HUSBANDRY

The room temperature was maintained at 20 ± 2° C with 30 - 70 % relative humidity.

The room was ventilated at the rate of approximately 15 air changes per hour.

Lighting was controlled to give 12 hours artificial light (8 a.m. - 8 p.m.) each day.

DIET

Water and standard pelleted feed (Amrut feeds ltd.) was freely available to the animals of both the groups *ad-libitum*.

EXPERIMENTAL PROCEDURE

A dose of 10% of the total diet of transgenic and non-transgenic foodstuff were incorporated into the feeding pellets of the rabbits of respective groups and fed for sixty days for sensitization with test proteins.

This study was carried out to determine the allergic reactions induced by test substance 'Bt Cottonseeds' in rabbits. The following battery of tests was conducted to determine the allergic potential of the 'Bt Cottonseeds'.

1. **Passive Cutaneous anaphylaxis (PCA)**

Principle- PCA is produced with the sera of allergic animal by challenging sensitized sites intradermally with intravenously injected antigen / allergen plus dye. Well-defined blue areas appear, indicating the sites of antigen-induced extravasations of fluid due to interaction with tissue fixed antibody.

Procedure- Naive animals were shaved on the back and flanks. Unblemished skin sites were selected and cleaned with 70% alcohol. 0.1 ml of test substance is injected intradermally using tuberculin syringe. After 24 hours, 0.6 ml of test substance was injected intravenously together with 0.4 ml of Evan's blue (25 % in physiological saline).

Observations- After 30-45 min. of intravenous injection, animals were killed and the lesions on the skin were evaluated for their intensity and diameter (Table-1).

TABLE-1 Observations (PCA)

Group	Animal No.	Area of dye Extravasations	
		Measurement (mm)	Intensity
Control Group (Non-Bt Cotton seeds)	Rabbit 1	5	+
	Rabbit 2	-	-
	Rabbit 3	5.1	+
	Rabbit 4	4.8	+
	Rabbit 5	4	+
	Rabbit 6	-	-
	Rabbit 7	-	-
	Rabbit 8	3.2	+
	Rabbit 9	-	-
	Rabbit 10	-	-
Treated (Bt Cotton seeds)	Rabbit 1	4	+
	Rabbit 2	-	-
	Rabbit 3	4.5	+
	Rabbit 4	-	-
	Rabbit 5	-	-
	Rabbit 6	-	-
	Rabbit 7	-	-
	Rabbit 8	5.2	+
	Rabbit 9	3.6	+
	Rabbit 10	-	-

* The test sample produces area of extravasations of about 15 – 20 mm with at least +++ intensity indicates positive reaction.

2. Prausnitz-Kustner (PK) test

PRINCIPLE-When normal skin is injected with reaginic serum, the reaginic antibodies become attached to the skin mast cells and the injected area of the skin acquires the specific skin reactivity towards challenged antigen / allergen.

PROCEDURE- Naive animals were shaved on the back and flanks. Unblemished skin sites were selected and cleaned with 70% alcohol. 0.05 ml of test substance was injected intradermally using tuberculin syringe. Control site were injected with 0.05 ml of physiological saline. After 24 hours, 0.05 ml of test substance was injected intradermally. 30-45 min. later animals were killed. The skin was opened so that the lesions could be evaluated. Diameter was measured and the intensity of the lesions was assessed.

OBSERVATIONS- After challenge phase wheal and flare formation (>3 mm) on the skin of animals was outlined and recorded (Table-2).

TABLE-2 Observations (PK Test)

Group	Animal No.	Wheal and Flare response (mm)
Control (Non-Bt Cotton seeds)	Rabbit 1	2
	Rabbit 2	-
	Rabbit 3	2.2
	Rabbit 4	2.3
	Rabbit 5	1.6
	Rabbit 6	-
	Rabbit 7	-
	Rabbit 8	1.5
	Rabbit 9	-
	Rabbit 10	-
Treated (Bt Cotton seeds)	Rabbit 1	1.3
	Rabbit 2	-
	Rabbit 3	3
	Rabbit 4	-
	Rabbit 5	-
	Rabbit 6	-
	Rabbit 7	-
	Rabbit 8	2.2
	Rabbit 9	2.7
	Rabbit 10	1.5

* A wheal and flare formation (>3 mm) in the skin indicates positive reaction.

3. Enzyme-linked Immunosorbent assay (ELISA)

PRINCIPLE-The IgE present in the serum is made to react with solid phase anti-IgE. The label, which in the present assay is an enzyme is taken up by the washed solid phase and is proportional to the IgE content of the sample under test and is then measured spectrophotometrically.

PROCEDURE- Test protein was adsorbed on microtitre plates. Test substance were pipetted into the walls of the microtiter plate together with ready to use anti-human IgE peroxidase conjugate. After a 30 minutes incubation at room temperature, the plate was rinsed with diluted wash solution. Then the substrate solution was pipetted and incubated for 15 minutes. The colour development was terminated by the addition of a stop solution provided in the kit.

OBSERVATIONS- Optical density of colour of plates were measured spectrophotometrically at the wavelength of 450 nm, using ELISA plate reader. The concentration of the antibodies is directly proportional to the intensity of the colour (Table-3a & 3b).

TABLE-3a Observations (ELISA)

Standard	I.U./ml	OD
1	0	0.037
2	5	0.269
3	25	0.421
4	100	0.811
5	250	0.831
6	1000	2.914

TABLE-3b Observations (ELISA)

Group	Animal No.	OD of Test – OD of Blank
Control	Rabbit 1	0.005
	Rabbit 2	0.003
	Rabbit 3	0.004
	Rabbit 4	0.006
	Rabbit 5	0.005
	Rabbit 6	0.007
	Rabbit 7	0.005
	Rabbit 8	0.004
	Rabbit 9	0.005
	Rabbit 10	0.003
Treated	Rabbit 1	0.007
	Rabbit 2	0.002
	Rabbit 3	0.004
	Rabbit 4	0.006
	Rabbit 5	0.005
	Rabbit 6	0.003
	Rabbit 7	0.005
	Rabbit 8	0.004
	Rabbit 9	0.007
	Rabbit 10	0.003

*Test is considered positive when the values are two fold or higher than the control

RESULTS

Under the conditions of the study, the test substance 'Bt Cottonseeds' was found to be negative for Passive cutaneous anaphylaxis test (PCA), Prausnitz- Kustner (PK) Test and Enzyme Linked Immunosorbent assay (ELISA). Therefore, the test substance 'Bt Cottonseeds' was found to be non-allergenic and is comparable to the 'Non-Bt Cottonseeds'. .

SHRIRAM INSTITUTE FOR INDUSTRIAL RESEARCH

(A unit of Shriram Scientific and Industrial Research Foundation)

An ISO - 9001:2000 Certified Institute

TEST CERTIFICATE

000046377

Issued to :
METAHELIX LIFE SCIENCES PVT. LTD.
PLOT NO. 3, KIADB 4TH PHASE,
BOMMASANDRA
BANGALORE - 560099KARNATAKA

Kind Attn: DR. M.J. VASUDEVA RAO , PRESIDENT

Sample Particulars:
One sample of "Bt Cottonseeds" was received for Allergenicity study.

J.O.No. TOX 346G
Reg.No. 4612570
Date 15-05-2007
GC-01 (REV-04)
Your Ref.No. --
Date

TEST RESULTS

Material Description : Non-Bt Cottonseeds (Sample-I)- Yellowish brown coloured powder
Bt Cottonseeds (Sample-II)- Yellowish brown coloured powder

Sponsor : Metahelix Life Sciences Private Limited
Plot no.3, KIADB 4th Phase, Bommasandra,
Bangalore-560 099, India.

Result

Allergenicity study

Under the conditions of the study, the test substance 'Bt Cottonseeds (Sample-II)' was found to be negative for Passive cutaneous anaphylaxis test (PCA), Prausnitz-Kustner (PK) Test and Enzyme Linked Immunosorbent Assay (ELISA). Therefore, the test substance 'Bt Cottonseeds (Sample-II)' was found to be non-allergenic and is comparable to the 'Non-Bt Cottonseeds (Sample-I) '.

The sample has been conducted as per DBT, Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant parts.

(Annexure enclosed)
****

DOR : 06-11-2006
DOC : 14-05-2007

Mittaggarwal
AUTHORISED SIGNATORY
(EMPLOYEE CODE: 6006)

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METAHELIX
Germinating Hope

PCR & ELISA CONFIRMATION OF BIOSAFETY COTTONSEED MATERIAL

Objective: Quality Control of the cottonseed material from cry1C-9124 based intrahirsutum hybrids to be used for the biosafety studies; despatched on 11th September 2006.

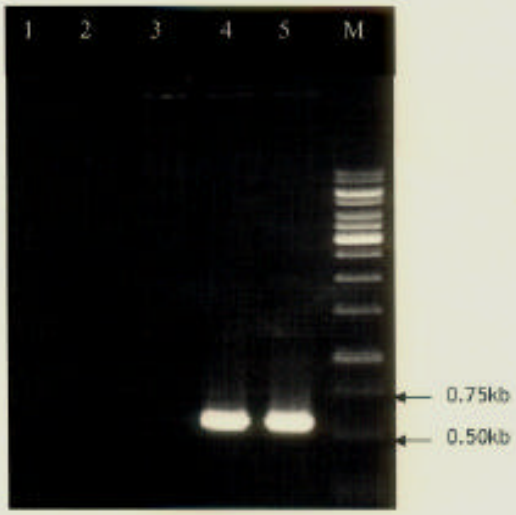
1. Confirmation of transgenic nature by PCR specific to the transgene
2. Confirmation of presence of Cry1C protein and its quantitation by ELISA

PCR confirmation

PCR was performed on Eppendorf Mastercycler Gradient machine with the following primers:

Upper:5'-CCT CGC CAT TCT TCG TGA TTC C
Lower:5'-GGT TGG CCT CCC TTC CGT AGA TA

1. H_2O CONTROL
2. -VE CONTROL (LEAF)
3. NON TRANSGENIC SEED DNA
4. TRANSGENIC SEED DNA
5. +VE CONTROL



EXPECTATION- 0.58 KB

Results and conclusion

As expected amplification from *cry1C* was observed in case of transgenic and positive control proving the presence of the gene. Water and negative controls were clear indicating the absence of gene.

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ELISA confirmation

Quantitative ELISA for Cry1C protein was performed using the Quantiplate kit for Cry1C (Envirologix, USA; Catalog No. AP 007) according to the manufacturer's protocol

Sl no	Entry ID	A_{450}	Cry1C concentration (µg/g on fresh wt)
1	Blank	0.09	NA
2	1 ppb standard	0.3	0.92
3	5 ppb standard	1.44	5.2
4	10 ppb standard	2.21	9.93
5	Nontransgenic	0.092	NA
6	Transgenic	2.9	13.08

Results

The absorbance value observed at 450nm for nontransgenic sample was nearly the same as blank and no colour development was observed in case of nontransgenic. Blue colour development was observed in case of transgenic samples indicating the presence of Cry1C protein.

Declaration

I hereby declare that the certificate of quality presented above is true to the best of my knowledge and is made on the basis of experiments carried out in our premises.

Vai. Ramanathan

Head - Genomics