General Protocols and Methods for Food and Feed Safety Testing

as per Indian Regulatory Requirements

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Quick Recap of earlier discussed topics …

GM-FOODS: NEW SUBSTANCES

- Genetic engineering can result in the **synthesis of new substances** in plants;
- Toxicological testing is required for such substances of **unknown safety**;
- It is **difficult to apply traditional toxicological testing** and risk assessment procedures to **food as a whole (WF)**;
- **An alternative approach** to be adopted for GM foods;

GM FOODS – ALTERNATE APPROACH

- **Goal is not establishing absolute safety** but to consider whether the GM food is as safe as its traditional counterpart, where such a counterpart exists.
- this comparative approach, embodied in the **concept of substantial equivalence**, is
- **not a safety assessment in itself,**
- it **represents the starting point** which is used to structure the safety assessment of a new food relative to its counterpart.

Quick Recap- India's Regulatory Framework

1

ICMR Guidelines for

The Safety Assessment of Foods Derived From Genetically Engineered Plants, 2008

2

Protocols for Food And Feed Safety Assessment of GE Crops, DBT, 2008

- **Toxicology studies are not considered necessary**
  - Where, there is **history of safe use**, or
  - where the new substance is **not present in the food.**
- **In other cases, use of conventional toxicology studies on the new substance will be necessary**. This may require
  - the isolation of the new substance from the GE plant, or
  - the production of the substance from an alternative source, in which case, the material has to be shown to be biochemically and functionally equivalent to that produced in the GE plant.

In 2008, DBT prepared and published five protocols under the title of "Protocols for Food and Feed Safety Assessment of GE Crops",

- Based on international best practices, guidance and peer reviewed publications available from the Codex Alimentarius Commission, the FAO, the WHO, the OECD and ILSI.

These protocols are:

1. Acute Oral Safety Limit Study in Rats or Mice
2. Subchronic Feeding Study in Rodents
3. Protein Thermal Stability
4. Pepsin Digestibility Assay
5. Livestock Feeding Study

- The results of these studies are be submitted by the applicant to the appropriate regulatory bodies (*i.e.*, RCGM and GEAC) as required.

Experiments intended to generate data to demonstrate the safety of foods derived from GE plants need to be,

- Designed and conducted in accordance with sound scientific concepts and principles,
- Data to be obtained using sound scientific methods and analysed using appropriate statistical techniques, where applicable.
- Conducted in accordance with Good Laboratory Practices (GLP), where applicable:
  - The sensitivity of all analytical methods should be documented and references to analytical methods made available.
  - Primary data should be made available to regulatory authorities upon request.
- Prior to making a submission, applicants are encouraged to consult with the concerned regulatory authorities for submission requirements for the primary whole food product derived from a GE plant.

DBT Protocols, 2008:
Acute Oral Safety Limit Study in Rats or Mice

- **ACUTE ORAL TOXICITY**
- those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.
- Source materials:
  - OECD 420: Acute Oral Toxicity – Fixed Dose procedure and the US-EPA OPTTS 870.1100 Health Effects Test Guideline.
- Limit Dose: a dose at an upper limitation on testing *e.g.*,
  - 2000 mg/kg body weight, or when this cannot be achieved,
  - the maximum possible dose based on the solubility of the protein.

DBT Protocols, 2008: Acute Oral Safety Limit Study in Rats or Mice

- For protein products that have a history of significant human dietary exposure, acute safety limit testing is not warranted.
- "if toxicity testing of a protein is considered necessary then acute exposure studies in laboratory animals should be sufficient, **since – if toxic – proteins are known to act via acute mechanisms**." [Sjoblad *et al*.(1992)]
- when a protein demonstrates no acute oral toxicity in high-dose testing using a standard laboratory mammalian test species, this supports
  - the determination that the protein will be nontoxic to humans and other mammals, and
  - will not present a hazard under any realistic exposure scenario, including long-term exposure.

Typical Study Plan: Acute Oral Toxicity Study-1

P R O T O C O L

Study Title

Recombinant ☐:

Acute Oral Toxicity Study in Mouse

(ICMR + DBT Guidelines)

Typical Study Plan: Acute Oral Toxicity Study-2

STUDY DESIGN AND ALLOTMENT OF ANIMALS

Group.	Dose (mg/kg)	No. of Male Mice	IDs. of Male Mice	No. of Female Mice	IDs. of Female Mice
G1 Vehicle control	≤ 10 ml/kg	5	Mb7721 to Mb7725	5	Mb7726 to Mb7730
G2	2000	5	Mb7731 to Mb7735	5	Mb7736 to Mb7740

Test Protein Information:

Physical state, purity, concentration, source, batch/lot reference number, and storage conditions.

When the test protein has been isolated from a source other than the GE plant, a characterization of the test protein and demonstration of equivalence with the plant-expressed form of the protein is required (normally as a separate study and report).

Typical Study Plan: Acute Oral Toxicity Study-3

- **TEST SYSTEM AND MANAGEMENT**
- **Test Species and Strain, justification for their selection:** :
  - Mouse; (or RAT)
  - **Mouse** is the species of choice because there is ample experience and background data available on this species. **Swiss Albino** strain has been selected due to availability of the strain and its historical control data at the test facility.
- **Sex:** Male and Female.
  - Females will be nulliparous and non-pregnant.
- **Age at start of study (at treatment):** 6 to 8 weeks
  - Before they are 9 weeks old
- **Body Weights prior to study:**
  - Body weight of groups being treated will not vary by ±20% of the average body weight of the groups of the same sex in the study.

Typical Study Plan: Acute Oral Toxicity Study-4

- **HOUSING AND FEEDING CONDITIONS**
- **Environmental conditions in experimental animal room:**
  - 100% fresh and filtered air, with 10 - 15 air changes per hour.
  - temperature between 19-25oC, relative humidity 30-70%. The illumination cycle set to 12 hours light and 12 hours dark.
- **Accommodation**: Single or group housed (max 5/sex/cage)
- **Diet :**
  - Extruded pelleted mouse feed provided ad libitum. The diet has been tested and certified to be free from undesired levels of contaminants.
  - Animals will be fasted for 3-4 hours prior to their treatment. Food will be offered 1-2 hours following dosing.
- **Water:**
  - Provided ad libitum, tested and certified for potability verified to be free from undesired levels of contaminants.

Typical Study Plan: Acute Oral Toxicity Study-5

- **PREPARATION OF ANIMALS**
- **Acclimation :**
  - At least for five days in the experimental room before start of Expt
  - Cages arranged to avoid spatial bias.
- **Randomization:**
  - Randomly selected for the study.
- **Identification:**
  - By cage tag and corresponding colour / tattoo body markings.
- **TEST PROTEIN DOSE PREPARATION – Vehicle**
- First consideration - aqueous solution/suspension of the test protein
- Second - solution/emulsion in oil (e.g.corn oil) and then by possible solution in other vehicles.
  - For vehicles other than water the toxic characteristics of the vehicle must be known.

Typical Study Plan: Acute Oral Toxicity Study-6

- **THE MAXIMUM DOSAGE VOLUME**:
  - not to exceed 10 ml/kg of body weight:
  - aqueous solutions : 20–25 ml/kg body weight
- **LIMIT DOSE:** 2000 mg/kg body weight OR the maximum possible based on solubility of protein.
- Prepare doses shortly prior to administration unless the stability of the preparation is known

- **TEST PROTEIN DOSE ADMINISTRATION**
- A single dose by gavage using a stomach tube or a suitable intubation canula.
- If a single dose is not possible, the dose may be given in smaller fractions over a maximum period of 12 hours normally or within 24 hours.

Typical Study Plan: Acute Oral Toxicity
Study-7: OBSERVATIONS

- **The observation period : at least 14-days.**
- **MORTALITY AND CLINICAL SIGNS OF TOXICITY**
  - **Observed after dosing** at least **once during the first 30 minutes**, and periodically during the first 24 hours, with special attention given during **the first 4 hours**, and **once daily thereafter**.
- **BODY WEIGHTS AND FEED CONSUMPTION**
  - **Individual weights**: shortly before dosing **(Day 0) and on Days 7 and 14.**
  - **Feed consumption** – measured at least weekly.
- **NECROPSY AND HISTOPATHOLOGY**
  - **Gross Necropsy** preformed for all animals (including those that die during the test or are sacrificed) and all gross morphological changes recorded.
  - Tissues with gross morphological changes will be subject to **histopathological examination**.

DBT Protocols, 2008: Subchronic Feeding Study in Rodents

- **Subchronic whole food feeding studies may be undertaken when:**
  - Compositional equivalence cannot be established and there is uncertainty over the nutritional and/or health impacts of the difference;
  - if the genetic modification affects multiple metabolic pathways and the potential impact on nutrition is not readily predictable;
  - if the genetic modification results in changes in levels of non-protein metabolites, or the synthesis of new ones;
  - or if other data are insufficient for a complete safety assessment.
- If feeding studies are warranted, it is recommended that a **90-day feeding study in rodents** be performed as the minimum to demonstrate safety.
- The 90-day whole food feeding study is **not intended to assess the potential toxicity of the protein expression product(s)** of the inserted gene(s) as this is accomplished via the acute oral toxicity study in rodents.

DBT Protocols, 2008:
90-day Subchronic Feeding Study in Rodents

- **Source material: OECD Test Guideline No. 408**
- **Purpose:** Assessment and evaluation of potential toxicity associated with a whole food derived from a GE plant.
- **Scope:**
  - provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth well into adulthood.
  - study will provide information on the major toxic effects, including possible target organs, and the possibility of cumulative effects.
  - This study should allow for the assessment of potential to cause neurotoxic, immunological or reproductive organ effects, which may warrant further in-depth investigation.

DBT Protocols, 2008:
90-day Subchronic Feeding Study in Rodents

STUDY DESIGN

Dose Group & Dose		**Dose mg / kg of diet**	**Treatment Period (90 Days i.e. 13 Weeks)**	
			Males	**Females**
G1 : Control Diet		0	10	10
G2	Test Diet	*	10	10
G3	Test Diet	*	10	10
G4	Test Diet	*	10	10
Total			40	40

* There can be ONE or MORE dose levels; The chosen dose level should be one that does not cause nutritional imbalance while, minimally, being comparable to anticipated human intake.

Test and control diets are administered to respective groups of test animals for a period of 90 days

DBT Protocols, 2008: 90-day Subchronic Feeding Study in Rodents

Parameters studied during repeated dose toxicity study

General Observation	Hematology	Clinical Chemistry
✓Mortality - daily twice ✓Clinical signs - once daily ✓Body weight - Weekly ✓Food Consumption –Weekly ✓Water Consumption – Weekly, if altered drinking activity	✓Hemoglobin (Hb) ✓Haematocrit (PCV) ✓Total Erythrocyte count (RBCs) ✓Total and Differential WBC count # ✓**General blood picture** ✓**Coagulation parameter** ✓Platelet count, or ✓APTT (Activated partial thromboplastin time) or ✓PT (Prothrombin time)	✓Alanine amino transferase (ALT) ✓Aspartate amino transferase (AST) ✓Alkaline phosphatase (ALP) ✓Gamma-Glutamyl Transferase (GGT) ✓Total Protein, Albumin, ✓Glucose ✓Cholesterol – Total ✓Urea nitrogen, Urea ✓Creatinine ✓Potassium (K) ✓Sodium (Na)

Parameters studied during repeated dose toxicity

Urine Analysis

- ✓Appearance
- ✓Volume
- ✓Specific gravity
- ✓pH
- ✓Protein
- ✓Glucose
- ✓Blood / Blood cells

Underlined organs will be trimmed of adherent tissue/fat and **weighed**, prior to preservation in fixative.

Histopathology

Gross lesions
Mesenteric lymph node
Axillary lymph node
Femur with Bone marrow
(and a bone marrow smear)
Eyes
Thymus
Trachea
Lungs
Heart
Aorta
Thyroid
Parathyroid
Oesophagus
Stomach
Duodenum
Jejunum
Terminal Ileum
Colon
Rectum

Histopathology – Contd.

Salivary glands
Liver
Gall bladder (mouse)
Pancreas
Spleen
Kidneys
Adrenals
Urinary bladder
Uterus
Testes / Ovaries
Prostate,
Seminal Vesicles
Epididymides
Skin
Mammary gland in female
Brain (cerebrum, cerebellum, midbrain)
Pituitary
Spinal cord (3 levels)
Skeletal muscle with
Sciatic nerve

Assessment of possible allergenicity of newly expressed proteins.

- At present, there is no definitive validated biological test involving animals that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore,
  - DBT recognises that an integrated, stepwise, case by case approach, should be used in the **assessment of possible allergenicity of newly expressed proteins**.
- This approach takes into account the preponderance of evidence derived from several types of information and data since no single criterion is sufficiently predictive.
- This includes, but is not limited to, the protocols
  - "**Protein Thermal Stability" and**
  - **"Pepsin Digestibility Assay**".

PROTEIN ALLERGENICITY

- Known protein allergens:
  - exhibit stability in the peptic and acidic conditions of the digestive system
  - tend to be stable to heat and processing
- Investigations on the thermal (heat) or processing stability of newly expressed proteins are part of a “weight-of-evidence” approach to assessing potential allergenicity.

DBT Protocols, 2008: Protein Thermal Stability Assays - Scope

- Heat denaturation does not necessarily result in protein degradation
- Heat stability assays are appropriate for proteins that exhibit a known enzymatic activity or biological activity for which there exist appropriate assay systems. Examples,
  - glyphosate resistant forms of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase,
  - herbicide detoxification enzymes such as phosphinothricin acetyltransferase (PAT), or
  - various insecticidal proteins (e.g., Cry1Ab, Cry1Ac, etc).
- Are not generally applicable to structural proteins or proteins without known enzymatic or biological activities that can be tested.

Protein Thermal Stability - Protocol

- Source Material:
- No fixed protocol; Several publications listed;
- some testing parameters included from a Joint FAO/WHO Expert Consultation Report on Allergenicity of Foods Derived from Biotechnology (2001).
- All newly expressed proteins to be tested
- A standard temperature regime should be followed wherever possible to aid the comparison of heat stability properties between different proteins.

Protein Thermal Stability – Brief Procedure

- Purified protein samples – make 1 mg/ml solution in a relevant buffer;
- Incubated separate samples at 25, 37, 55, 75 and 95°C for up to 30 minutes.
- Follow by rapid cooling on ice;
- Perform assays of the biological activity of the protein and compare to control samples of the protein maintained on ice.
  - Perform quantitative assays where possible, to enable determination of a threshold level of 10% of the activity of the untreated sample.
- **Interpretation: of Protein stability**
- **Stable** - those with more than 50% biological activity remaining.
- **Partially stable** – those with between 50 and 10% biological activity
- **Labile – those showing less that 10% biological activity**
  - there needs to be consideration of the relevance of the particular temperature to human exposure, for example, whether the food is processed or cooked before consumption.

PEPSIN DIGESTIBILITY ASSAY

- Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system in order to reach and pass through the intestinal mucosa to elicit an allergic response (Metcalfe *et al., 1996; Taylor et al., 1987; Taylor,* 1992).
- 'Digestion stability' is one component of a comprehensive weight-of evidence approach to assessing allergenic potential (Codex, 2003).
- **DBT Protocol's Source Material:**
- No fixed protocol; Several publications listed;
- some testing parameters included from a Joint FAO/WHO Expert Consultation Report on Allergenicity of Foods Derived from Biotechnology (2001).

Pepsin Digestibility Assay – Brief Method

- **Test system:** in vitro digestion using porcine pepsin in simulated gastric fluid (SGF)
- **Assay is performed under standard conditions**
  - 10 units of pepsin activity per microgram of test protein.
  - Two pH conditions - pH 1.2 and pH 2.0 and Temperature : 37°C
- **Sampling Times** of the digestion reaction mixture : 0, 0.5, 1, 2, 5, 10, 20, 30, 60 minutes.
- Activity of pepsin is quenched by **neutralization** with carbonate buffer and SDS-PAGE loading buffer, then heating to more than 70°C for 3 to 5 minutes.
- Samples are **separated by SDS-PAGE** and stained with Coomassie or colloidal blue to evaluate the extent of digestion.
- **Western blot analysis** of the digested samples with antibodies specific to the test protein is used to illustrate specific digestion of the target protein and the presence or absence of lower molecular weight digestion products.

Pepsin Digestibility Assay – Controls

- **Control samples in the Assay:**
  - test protein in SGF reaction mixture without added pepsin, T=0 min;
  - test protein in SGF reaction mixture without added pepsin, T=60 min;
  - SGF with added pepsin but without test protein, T=0;
  - SGF with added pepsin but without test protein, T=60; and
  - **a 10% test protein sample** and quenched pepsin without SGF reaction mixture (to verify detectability of at least 10% of the original protein concentration).
- **Stability of the protein is the time required to reach 90% digestion**, estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard.
  - Stable (or partially stable) intermediate proteolytic fragments - any new bands with MV > 3,000 approx.
  - Western blot analysis would identify if any of the intermediate products are derived from the test protein.

Pepsin Digestibility Assay – Interpretations

- **Interpretations:**
  - **Stable Proteins** – those with more than 10% stainable full-length protein band remaining at > 30 to 60 minutes
  - **intermediate stability** - Proteins reduced to < 10% stainable band at 5 to 30 minutes
  - **Labile (rapidly digested) Proteins** – those reduced to < 10% stainable band by 2 minutes.
- Most non-allergenic food proteins are digested by approximately 30 seconds, while major food allergens are stable, or produce pepsin-stable fragments that are detectable for from eight to 60 minutes. Ref: A review of the digestibility assay by Bannon *et al. (2002) and by Thomas et al. (2004).*

Assessment of possible allergenicity…

- DBT is currently developing additional protocols for
  - specific serum screening and
  - amino acid sequence homology comparisons.
- As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy.
  - These include targeted serum screening and the use of animal models.

Livestock Feeding Study

- The aim of livestock feeding trials is primarily to evaluate the nutritional parameters (*e.g.*, wholesomeness and nutrient bioavailability) of the food and/or feed under relevant circumstances.
- Livestock feeding trials are not designed, nor are they sufficiently sensitive, to evaluate the potential toxicity of individual proteins or the potential toxicity associated with the whole food.
- These latter questions are more appropriately addressed through 14-day acute toxicity studies, in the case of individual proteins, or, if warranted, whole food 90-day sub-chronic feeding studies in rodent species.

Livestock Feeding Study - Scope

- **Two situations** in which livestock feeding trials may be of value:
  - (1) **if significant compositional differences** are observed between the GE food and its comparator, then feeding trials may be used **to investigate the biological significance of such differences**; and
  - (2) in the case of a GE **food with enhanced nutritional characteristics**, livestock feeding trials may be used **to demonstrate that the expected nutritional benefit** is achieved.

Livestock Feeding Study – Principle

- The GE plant product (*e.g., grain, forage, meal etc) is*
  - incorporated into livestock feed rations and provided as feed to an appropriate livestock species,
  - for a period of time approximating a normal production cycle.
- Measurements
  - body weight and feed consumption - taken periodically,
  - Animals slaughtered at the end of the study and carcass yield data collected.
- Control animals receive diet formulated with plant product from the conventional comparator.
- The experimental design of the study should be sufficient to detect, at $P<0.05$, a 5–10% difference in animal performance.

Livestock Feeding Study – Brief Protocol-1

- **Choice of Test Species - Broiler Chicken**
  - Offers advantage of significantly higher exposure over nearly the complete life span of the animal
  - Rapidly gain weight, hence sensitive to any change in nutrient supply or the presence of toxic elements in their feed.
  - provide a genetically homogeneous population;
  - can be used in relatively large numbers to increase the statistical power of the experiment
- **Test and control diets:**
- Each diet should contain the same level of incorporation of plant material derived from either GE or control non-genetically engineered plants, and this level should not result in nutritional imbalance.

Livestock Feeding Study – Brief Protocol-2

- **The GE and control plant material** should be
  - Grown under identical environmental conditions and harvested and processed at the same time, using the same equipment and under the same conditions.

- **Nutrient analysis** should be carried out on
  - the harvested plant material, any processed products, and on the final formulated test and control diets.
  - The nutrients to be analyzed are those that are important for meeting the requirements of the recipient livestock or poultry species.
- Knowing the nutrient content is critical to formulating the final prepared feed as nutrient deficiency or imbalance may result in decreased animal performance.
- In the case of a GE crop with enhanced nutritional characteristics, additional compositional analysis may be warranted.
- Diets should be processed to a physical form (meal, pellets, crumbles, etc); test and control diets should be fed in the same form.

Livestock Feeding Study – Brief Protocol-3

- **The GE and control plant material** should be
  - Grown under identical environmental conditions and harvested and processed at the same time, using the same equipment and under the same conditions.

- **Nutrient analysis** should be carried out on
  - the harvested plant material, any processed products, and on the final formulated test and control diets.
  - The nutrients to be analyzed are those that are important for meeting the requirements of the recipient livestock or poultry species.
- Knowing the nutrient content is critical to formulating the final prepared feed as nutrient deficiency or imbalance may result in decreased animal performance.
- In the case of a GE crop with enhanced nutritional characteristics, additional compositional analysis may be warranted.
- Diets should be processed to a physical form (meal, pellets, crumbles, etc); test and control diets should be fed in the same form.

Livestock Feeding Study – Brief Protocol-4

- **Test Parameters**
  - Survival and Clinical Signs
  - Necropsy – gross pathology
  - Body weight and feed consumption
  - Water consumption
    - considered when drinking activity may be altered
  - Carcass measurements
    - weights of dressed carcass, fat pad, drums, thighs, wings, *Pectoralis major, and Pectoralis minor.*
  - Other measurements

Review and Updation of Protocols and Requirements

- DBT intends to review scientific literature and international standards on a regular basis to ensure that the scientific guidance used to support the safety assessment is kept abreast and up-to-date with internationally accepted best practices.

Thank you !!

Questions are Welcome !!

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