

DETERMINATION OF THE PERSISTENCE FREQUENCY OF DIFFERENT COMPONENTS OF THE CRY1AC TRANSGENE CASSETTE IN MAMMALIAN TISSUES

Ali M Sajjad¹, Afroz Yasmeen¹, Sarfraz Ahmad¹, Usman Sagheer¹

¹Institute of Molecular Biology and Biotechnology, The University of Lahore, Pakistan

Abstract

The safety of the transgenic feeds for the mammals is a long standing issue which casts a shadow over their acceptance by the farming community in large. The present study was designed to foresee the fate of the components of the transgene cassette (MON531 event) in mammalian digestive system. The objective includes adopting an in vivo approach to study the possible partial degradation of the consumed transgene in mice. The mice were fed upon a feed mix containing 50% transgenic flour from crushed Bt cotton seeds. Mice were slaughtered periodically to draw tissue samples of stomach, intestine, blood, liver, kidney, heart and brain. The purified DNA when subjected to PCR under primers for different nucleotide sequences of the transgene cassette revealed the presence of cry1Ac gene sequence and tnos in intestinal tissues only. However there was no evidence of absorption through the epithelial lining into blood nor other body organs. This study reveals the possible partial degradation of the transgenic nucleotides in the acidic fluid of mammalian stomach.

Key words: genetically modified cotton; cry1Ac gene; swiss albino mice; feeding trial

1. INTRODUCTION

Bt cotton has been genetically modified to express cry gene(s) from *Bacillus thuringiensis* (Bt). It is resistant to attack by Lepidopteran insect pests, as it expresses the Cry1Ac protein. Although the bacterial Cry1Ac protein has been extensively used as an organic insecticide (Betz et al, 2000), however its expression in transgenic cotton could potentially alter its structure which may render it allergenic or otherwise harmful upon ingestion (Prescott et al, 2006) as the cotton seeds are used as a component of feed for cattle.

A large number of farmers and shepherds use cotton seed meal as a component of fodder for their cattle. Cotton seed cake is made from cotton seeds after extracting oil from the cotton seeds. It is a good source of nutrition and has long been used as a highly economic protein- concentrated animal feed. Large scale cultivation of Bt cotton exposes a large population of cattle and dairy animals to the transgenic nucleotide sequence present in the feed which has long been a source of scientific controversy with one of the main concerns relating to potential health effect (EFSA, 2009). Concerns include potential gene transfer from GM plant to organs or indigenous micro biota of animals.

2. BACKGROUND

The gastrointestinal tract (GIT) is the main portal for entry of foreign macro-molecules in mammals. The epithelial cells of the lumen are continually exposed to dietary DNA and therefore provide a possible route for uptake of food/feed-associated DNA and proteins from the GIT (Aurora et al, 2011). It is important to understand the stability of DNA in feed because some degree of persistence and integrity of DNA in feed products is a prerequisite for the exposure of the GIT to dietary DNA. The fragments of *P35S* and *cp4epsps* were found in the tissues (liver, kidney, heart and muscle) of goats fed on transgenic maize carrying *cry1Ab* gene (Swiatkiewicz et al, 2011). DNA fragments of high-copy-number plant genes (usually from the chloroplast) can be found in some animal tissues, and have been detected in the blood, muscles, and internal organs of broiler chickens, calves and pigs and in muscles, milk and blood of cattle (Tony et al, 2003; Reuter et al, 2003; Nemeth et al, 2003). Martín-Orúe et al, (2002) evaluated the degradation level of recombinant DNA from GM soya and GM maize using in vitro simulations of human digestion. The results indicated that some recombinant DNA may survive the passage through the stomach and small bowel.

These studies show that small fragments belonging to GM plant nucleotide sequences can be detected in the digestive tract epithelial lining which might travel into interior organs such as blood, liver, brain etc. Cultivation of Bt cotton has raised fears among the shepherds and other livestock owners over the safety of Bt cotton plants and their by-products. Therefore, the present study was carried out to evaluate the persistence of GM nucleotides

in different tissues of the test animals (mice) by mixing Bt cotton seed meal in their diet. The mice were fed with transgenic cotton meal (event MON531) carrying the transgene *cry1Ac* with the promoter sequence *CAMV35S* and terminator sequence of *nos*. The data obtained will help to conduct a safety assessment with regard to the transfer of genetic elements following feed consumption and to evaluate if the use of GM plants in livestock feed presents a higher risk than conventional plant use.

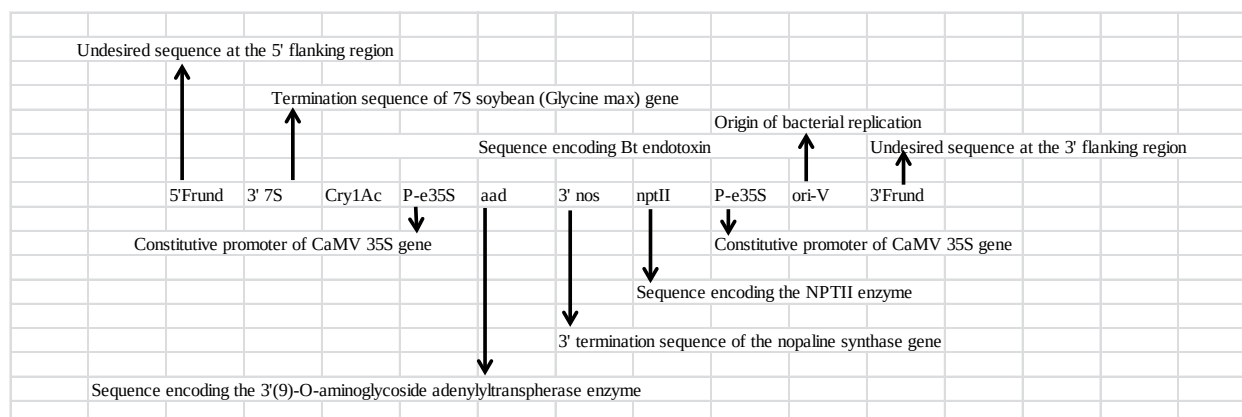


Fig 1. Event specific structure of MON531 Bt cotton source: Biosafetyscanner.org

3. MATERIAL AND METHODS

The transgenic cotton (MON531 event) is developed by introducing the *cry1Ac* gene from *Bacillus thuringiensis* (Bt) and has the ability to resist the attack from lepidopteran insect pests especially *Helicoverpa armigera* (Hubner). Bt cotton varieties Ghaury, BT456, SGA1, SL1, BT666, BT886, CM555 and CM996 were accessed from the seed market Multan (Pakistan). The seeds were produced in the late season of 2012 in Punjab, Pakistan. All shipping and handling was conducted to protect the freshness and quality of the Bt cotton seeds. On arrival at the Institute of Molecular Biology and Biotechnology (IMBB), the seeds were stored at 5°C before grinding and subsequent storage at 18°C until flour making. The presence of the *Cry1Ac* transformation cassette was confirmed by DAS ELISA kit (Agdia).

Three colonies of four weeks old swiss albino mice (24 mice/ colony) were obtained periodically each at the end of one culling session after two months gap. In one colony, four mice were fed on conventional diet while the remaining 20 mice were reared upon GM cotton meal + regular diet in different proportions with GM feed content ranging from 60-90%. Test and control animals were housed in separate cages each of size 28cm x 38cm x 16.6cm. Mice were obtained from the mice rearing unit established at IMBB, UOL Pakistan. The animals were housed at 22 ± 1 °C, relative humidity 55 ± 5% and electric light from 09.00 to 21.00. The mice were allowed free access to both food and water.

The feeding trials spanned over 12 months with the sacrificing of 5 colonies of mice each comprising of 24 mice. Animal experiments and housing procedures were performed in accordance with the biosafety regulations set by internal biosafety committee of IMBB. Before slaughtering the animals were kept on fasting for 12 hours to avoid any presence of the transgenic food elements in the digestive tract during sampling and DNA isolation.

At terminal sacrifice, the animals were anaesthetized by injecting ketamine (1/10 volume in distilled water) and killed by dissection for the tissue samples of stomach, intestine, liver, kidney, heart and brain. Blood samples were taken from the heart and collected in EDTA and heparin coated tubes. In total, 120 mice were slaughtered. One mice from conventional feed group and four mice from the GM feed group were slaughtered after every 15 days. It resulted in a total of 840 samples. The overall slaughtering period for one colony lasted for 2 months. Each of the samples was later subjected to conventional PCR and gel electrophoresis to confirm the presence of transgenic nucleotide sequence in the body tissues/blood.

During the dissection procedure special care was taken to avoid accidental contamination from the environment or from the surgical tools. The working place and the tools were accurately cleaned with a 5% hypochlorite solution rinsed in sterile water.

For DNA extraction from mice tissue, Purelink Genomic DNA extraction kit (Invitrogen) was used according to the provided protocol. Prior to PCR, the extracted DNA was diluted in molecular biology grade water to give 500 ng DNA per 5 μ l. PCR master mix (Invitrogen) and 100bp ladder (Invitrogen) were used.

The Primers used for the identification of target nucleotide sequence were as follows:

Table 1. List of the construct specific primer pairs for qualitative PCR

Nr	Target sequence	Sequence (5'—3')	Amplicon size (bp)
1	<i>p-35s</i>	F: GCTCCTACAAATGCCATCA	195
		R: GATAGTGGGATTGTGCGTCA	
2	<i>cry1Ac</i>	F: GCCAATGCCTCGTGATTGTTCTCTGC	280
		R: GATTTGCGAGGCTGGCCAGCTCCACG	
3	<i>t-nos</i>	F: GAATCCTGTTGCCGGTCTTG	180
		R: TTATCCTAGTTTGCGCGCTA	
4	<i>nptII</i>	F: GAGGCTATTTCGGCTATGACTG	700
		R: ATCGGGAGCGGCGATAACGTA	

Each type of tissue DNA was used with all four primer pairs individually during PCR. PCR analysis of the DNA from dissected tissues was carried out using the published primers (Oraby et al, 2005; Randhawa & Firke, 2006; Ceron et al, 1995; Litao et al, 2005) for the construct specific nucleotide sequences i.e. *cry1Ac* and *P35S*, *TNOS* and *nptII*. The primer sequences and their amplicon sizes are shown in Table 1. All the primers were synthesized by Vivantis Technologies. The analysis of very small DNA quantities can be performed by qRT PCR technique which allows an accurate and sensitive high throughput. To find out sensitivity in detection of GMO's, qRT PCR was performed in iCycler (Bio Rad) with a final volume of 25 μ l (20 μ l of water, 0.8 μ M of each primer, 3 μ l of genomic DNA and 25 μ l of SyberGreen PCR Super Mix Universal (Invitrogen). The reactions were carried out at 95°C for 3 min, followed by 50-60 cycles of amplification: denaturing at 94°C for 40 sec, annealing at 54°C (*nptII*, *P-35S* and *nos*) and 58°C (*cry1Ac*) for 30 sec and extension at 72°C for 40 sec. The PCR amplicons were analyzed on 2% agarose /ethidium bromide gel (Vivantis) along with 100bp DNA ladder (Invitrogen), visualized by direct observation on a UV trans-illuminator and the images were recorded using a gel documentation system (Bio Rad). During gel electrophoresis run, each well contained 20ul of sample. Gel was electroplated for one hour at 30mA and 100v. The gel was photographed in Gene Genius bio-imaging Gel Documentation system.

4. RESULTS & DISCUSSION

DNA isolations were performed on each tissue and tested with quantitative real time PCR for the presence of four target gene fragments (*P35S*, *cry1Ac*, *Tnos*, *nptII*). The presence of the transgenic nucleotide sequence in mammalian tissue DNA was confirmed only for certain individuals of the colonies 2-5 (Fig 2, 3, 4, 5). The highest detection frequency was observed for the individuals fed upon transgenic feed content of 70-90%.

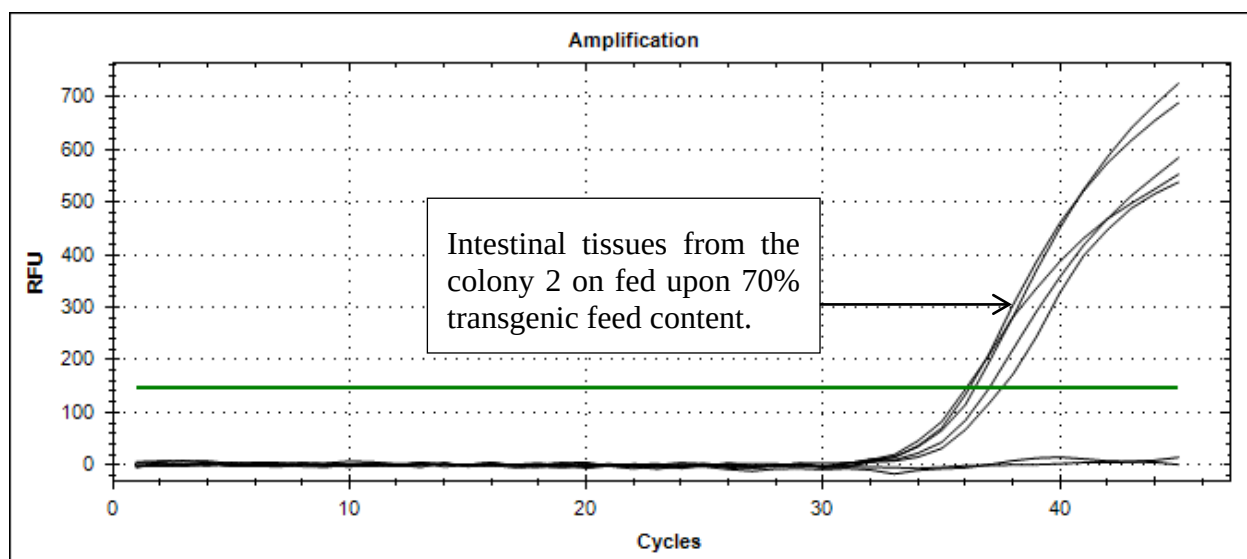


Fig 2. Amplification curve for Tnos for the tissue samples from colony 2 for the individuals fed upon 70% transgenic feed content. No amplification curves were observed for samples from control animals fed upon conventional feed.

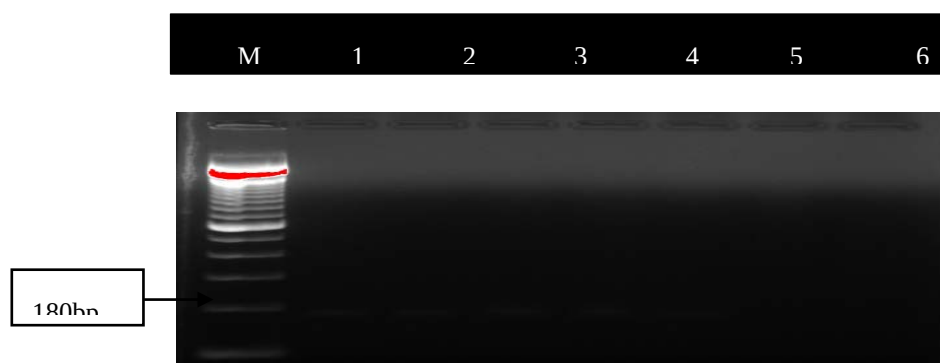


Fig 3. The fragments of *tnos* are confirmed for their presence through gel electrophoresis: M- DNA marker; 1- MON511 cotton, 2-5 intestinal tissues (colony 2); 6, 7- intestinal tissues from the control animals.

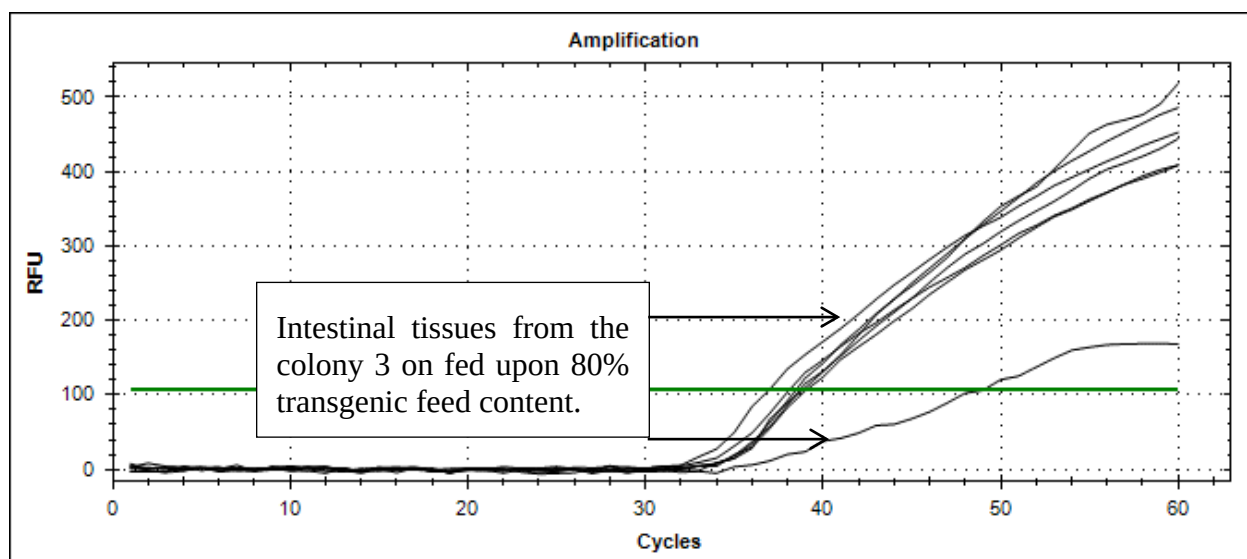


Fig 4. For *cry1Ac*: M- DNA marker; 1- MON531 cotton, 2-7 intestinal tissues (colony 3);

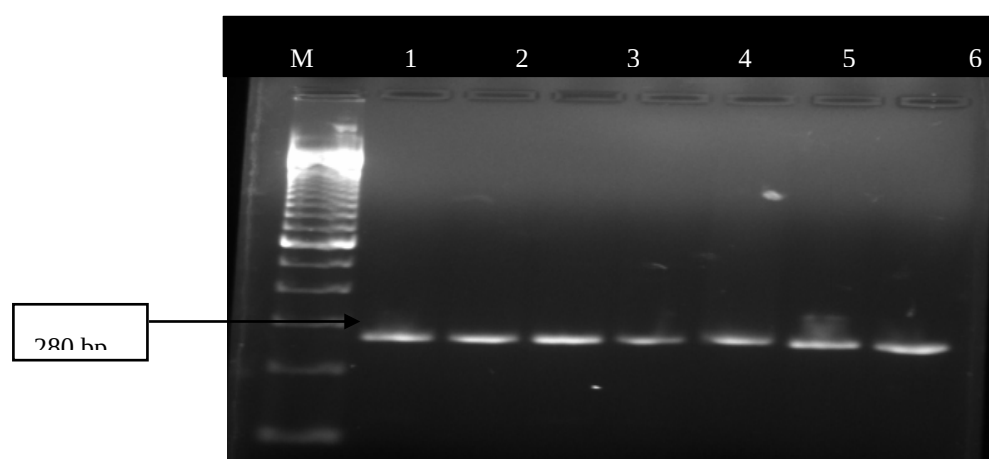


Fig 5. The fragments of *cry1Ac* are confirmed for their presence through gel electrophoresis: M- DNA marker; 1- MON531 cotton, 2-7 intestinal tissues (colony 3);

Table 2: The detection frequency of various nucleotide sequences in different tissues of the animals dissected

Colony No	Feed composition	CF=conventional feed TF=Transgenic feed		
		Total number of samples collected	Nucleotide sequence detected	Frequency of detection
1	Conventional feed	175	None	0

2	40% CF+60%TF	175	<i>t-nos; cry1Ac</i>	7
3	30% CF+70%TF	175	<i>t-nos; cry1Ac</i>	12
4	20% CF+80%TF	175	<i>t-nos; cry1Ac</i>	12
5	10% CF+90%TF	175	<i>t-nos; cry1Ac</i>	9

The transgene was confirmed for presence only in intestinal tissues whereas none was observed in any of the tissues of mice from any colony. The intestinal tissue samples from colonies 2-5 showed the presence of various parts of the transgene

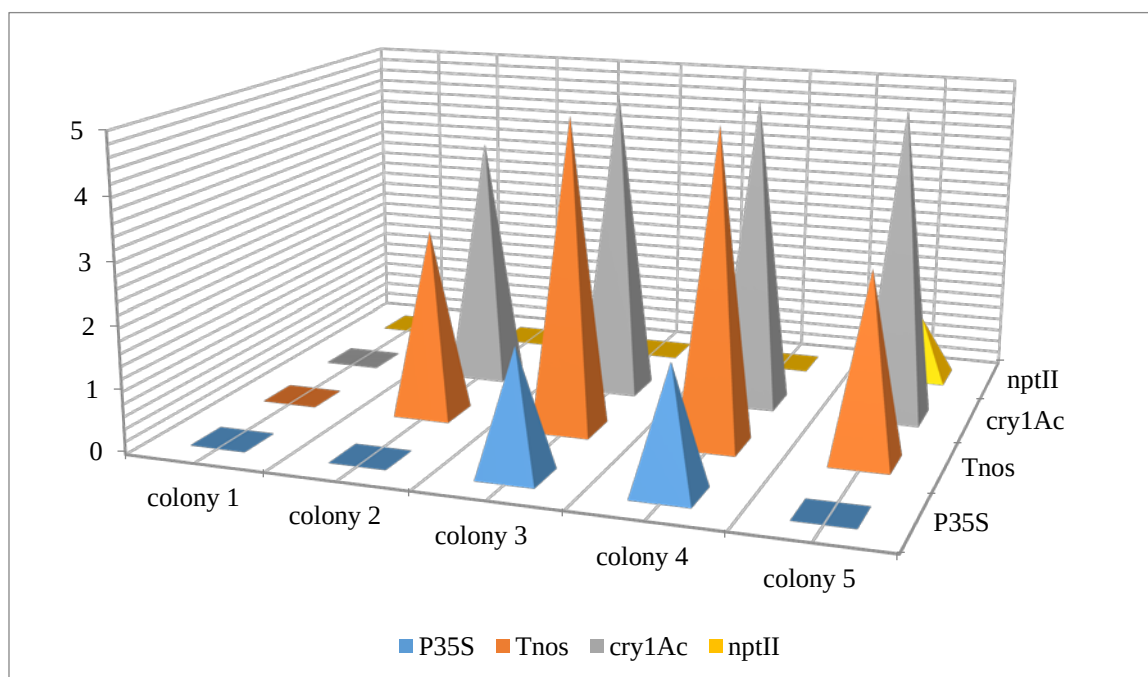


Fig 6. Frequency of presence of transgene segments in intestinal tissues of colonies 1-5.

DF=Detection frequency is the number of positive results among the total number of samples

TS=Total number of samples examined

A sufficiently large number of samples i.e. 840 were obtained from the mice where only 5% showed signs of the persisting transgene (fig. 7).

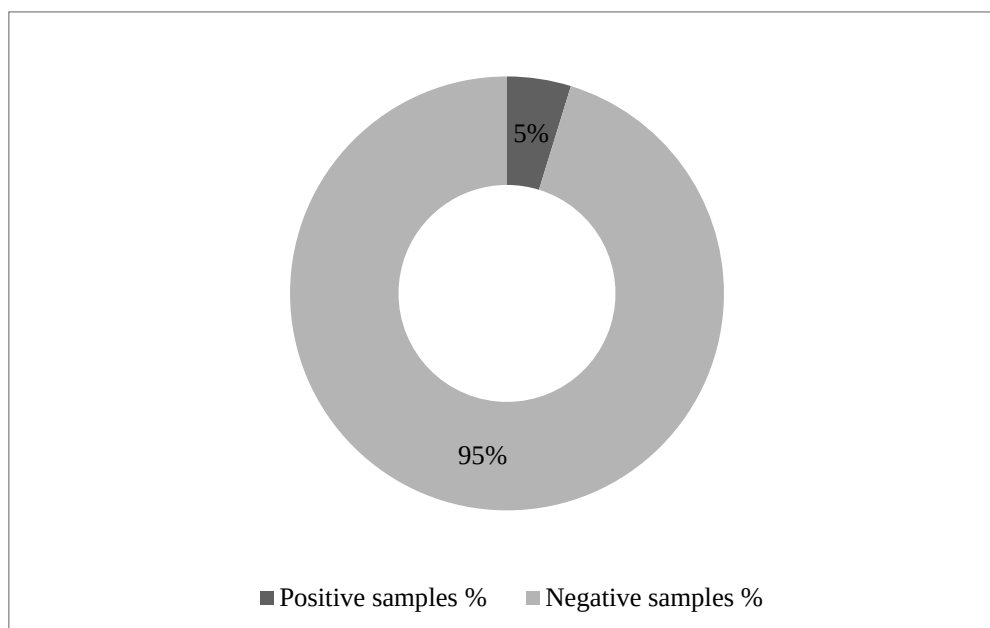


Fig 7. Percentage of the samples with/without transgene's presence

DNA is a regular component of the animal diet, so ingestion of nucleic acids via feed is a naturally occurring process, and farm animals consume in the order of grams of foreign DNA per day (Flachowsky et al, 2005). Ingested DNA is broken down in the gut to smaller monomers by the mechanical process of mastication, acid hydrolysis and pancreatic and intestinal enzymatic activity of nucleases (Beever & Kemp, 2000). Results of experiments with pigs indicated that fragments of endogenous, chloroplast DNA from maize could be detected in intestinal contents up to 12h (Klotz et al, 2002) or 72 h after the last feeding. Small fragments of DNA that have not been totally hydrolyzed by the action of pancreatic and intestinal enzymes may be absorbed by intestinal mucosa and transported to different parts of the animal body. Results of some experiments carried out on poultry proved that there is the possibility of transfer to body tissues of highly fragmented endogenous plant DNA, especially fragments present in cells at high concentrations (multi-copy genes). In a study with broilers the high-copy number maize-chloroplast *ivr* gene (226 bp) was amplified in samples of gizzard, small intestines (weak signal), blood, muscle (weak signal), liver and spleen, both from birds fed conventional and transgenic maize (Aeschbacher et al, 2005).

However, the present study confirms the presence of fragments of transgenes only in intestinal samples, but not in subsequent section of intestinal tract, liver tissue, kidney, heart and any other part of mice. Similar results were reported by (Deaville & Maddison, 2005) who showed that transgenic DNA from RR soybean meal or Bt maize was detectable in broiler gizzard, but not in intestinal digesta, blood and selected tissues. For comparison, in a study with fallow deer (*Dama dama*) fed Bt maize, small chloroplast- and maize-specific DNA fragments were detectable in contents of rumen, abomasum, jejunum, caecum and colon, but no fragments of the recombinant *cry1Ab* gene were detectable in gastrointestinal contents, indicating complete digestion of the GM maize (Guertler et al, 2008).

In our study transgenic DNA of Bt cotton was not detected in blood and tissues i.e. kidney liver, lungs, heart and brain of mice. The results are confirmed by the studies of Jennings et al (2003), who reported that small sized fragments of transgenic (211 bp) or endogenous DNA (213 bp) were not detectable in breast muscle of chickens fed with diet containing Bt (MON 810) maize. Similarly, transgenic DNA from GM event CBH 351 (Star Link) hybrid maize was not found in the tissues (blood, livers and muscle) of broilers (Yonemochi et al, 2002). Tudisco et al (2006) reported the fate of transgenic Bt gene in poultry organism was determined also in a long-term feeding study with quails. The above authors pointed out that a short fragment of transgene (211 bp) was detectable in the gizzard digesta and along the whole gastrointestinal tract, but not in tissues (muscle, liver, stomach, spleen, kidney, heart) and eggs from quails fed for ten generations with a diet containing high levels of Bt maize. The metabolic fate of DNA from conventional or Bt maize was determined in broiler chickens also by (Einspanie et al, 2001). They found short fragments (<200 bp) of plant chloroplast-specific (non-transgenic) DNA in muscle, liver, spleen and kidney, but no transgenic fragments of Bt gene were detectable in samples of

these tissues. In a recent study with zebra fish (*Daniorerio*) fed upon a diet containing GM maize and soybean, fragments of transgenic DNA from MON 810 maize were found, while transgene fragments from RR soybean were not detectable in organs (Sissener et al, 2010).

The differences observed in different animal experiments in degradation, digestion and uptake of conventional or transgenic DNA fragments can be probably dependent on nucleotide sequences, methylation patterns or simply the feed matrix.

5. CONCLUSION

The results of this study with transgenic mice showed incomplete digestion in the gastrointestinal tract where the tissue samples from the small intestine validated the presence of P35S, cry1Ac and Tnos. However there was no detection of transgenic nucleotide fragments in stomach, liver, kidney, heart and brain as well as blood samples. The research although carried out in strict scientific scrutiny and protocols elucidates the importance of many such studies to be carried out for a variety of the transgenic foods being marketed. It will help to increase the acceptance of such foods/fodder in general public.

REFERENCES

- Aeschbacher K., Messikommer R., Meile L., Wenk C. (2005) Bt176 corn in poultry nutrition: physiological characteristics and fate of recombinant plant DNA in chickens. 84: p. 385–394.
- Aurora Rizzi, NRDDCS. Lise Nordgrd , Kaare Magne Nielsen. (2011) The Stability and Degradation of Dietary DNA in the Gastrointestinal Tract of Mammals. Implications for Horizontal Gene Transfer and the Biosafety of GMOs.
- Authority, E.F.S.A. (2009) EFSA and GMO risk assessment for human and animal health and the environment. November 2009.
- Beever D E., Kemp F. (2000) Safety issues associated with the DNA in animal feed derived from genetically modified crops: a review of scientific and regulatory procedures. 70: p. 197–204.
- Betz FS, H.B., Fuchs RL. (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. *RegulToxicolPharmacol*, 32: p. 156–173.
- Deaville ER., Maddison BC. (2005) Detection of transgenic and endogenous plant DNA fragments in the blood, tissues, and digesta of broilers. 53: p. 10268–10275.
- Einspanie R., Klotz A.,Krat J.,Aulrich K., Poser R., Schwagele F., Jahreis G.,Flachowsky G. (2001) The fate of forage plant DNA in farm animals: a collaborative casestudy investigating cattle and chicken fed recombinant plant material. 212: p. 129–134.
- Event specific structure of MON531 Bt cotton. Available at:
<http://en.biosafetyscanner.org/schedaevento.php?evento=30>
- Flachowsky G.,Halle I.,Aulrich K. (2005) Long term feeding of Bt-corn – a ten-generation study with quails. 59: p. 449–451.
- Guertler P.,Lutz B.,Kuehn R.,Meyer HHD.,Einspanier R.,Kile rmann B., Al-brecht C. (2008) Fate of recombinant DNA and Cry1Ab protein after ingestion and dispersal of genetically modified maize in comparison to rapeseed by fallow deer (*Dama dama*). 54: p. 36–43.
- Jennings J.C.,Albee L.D.,Kolwyck D.C.,Surber J.B.,Taylor M.L., Hartnell G.F., Lirette R.P.,Glenn K.C. (2003) Attempts to detect transgenic and endogenous plant DNA and transgenic protein in muscle from broilers fed YieldGard corn borer corn. *Poultry Sci*, 82: p. 371–380.
- Klotz A.,Mayer J.,Einspanier R. (2002) Degradation and possible carry-over of feed DNA monitored in pigs and poultry. 214: p. 271–275.
- Martín-Orúe SM, O.D.A., Ariño J, Netherwood T, Gilbert HJ, McMathers JC. (2002) Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. (87): p. 533-542.

- Nemeth, A., Wurz, A., Artim, L., Charlton, S., Dana, G., Glenn, K., Hunst, P., Jennings, J., Shilito, R., Song. (2003) Sensitive PCR analysis of animal tissue samples for fragments of endogenous and transgenic plant DNA. *J. Agric. Food Chem*, 52: p. 6129–6135.
- Oraby H.A.S., Hassan A.A. Mossallam A.A.A. (1980) Screening food products for the presence of CaMV 35S promoter and NOS 3' terminator, *J Sci Food Agric*, 85:
- Randhawa G.J., Firke P.K. (2006) Detection of transgenes in genetically modified soybean and maize using polymerase chain reaction, *Indian J Biotechnol* 5: 510-513
- Ceron J., Ortiz A., Quintero R., Guereca L., Bravo A. (1995) Specific PCR primers directed to identify cryI and cryIII genes within a *Bacillus thuringiensis* strain collection, *Appl Environ Microbiol*, 61: 3826-3831
- Litao Yang., Aihu Pan., Kewei Zhang., Jinchao Guo., Changsong Yin., Jianxiu Chen., Cheng Huang., Dabing Zhang. (2005) Identification and quantification of three genetically modified insect resistant cotton lines using conventional and taqman real-time polymerase chain reaction methods, *J Agric Food Chem* 53(16): 6222-6229
- Prescott VE, H.S. (2006) Genetically modified plants and food hypersensitivity diseases: Usage and implications of experimental models for risk assessment. 2006(111): p. 374–383.
- Reuter, T., Aulrich, K. (2003) Investigations on genetically modified maize (Bt-maize) in pig nutrition: fate of feed-ingested foreign DNA in pig bodies. *Food Res. Technol.* 216: p. 185–192.
- Sissener N.H., Johannessen L.E., Hevroy E.M., Wiik-Nielsen C.R., Berdal K.G., Nordgreen A., Hemre G.I. (2010), Zebrafish (*Danio rerio*) as a model for investigating the safety of GM feed ingredients (soya and maize); performance, stress response and uptake of dietary DNA sequences. 103, p. 3–15.
- Swiatkiewicz M., Hanczakowska E., Twardowska M., Mazur M., Kwiatek K., Kozaczynski W., Swiatkiewicz S., Sieradzki M. (2011) Effect of genetically modified feeds on fattening results and transfer of transgenic DNA to swine tissues. *Pulawy*, 55: p. 121–125.
- Tony, M.A., Butschke, A., Broll, H., Grohmann, L., Zagon, J., Halle, I., Danicke, S., Schauzu, M., Hafez, H.M., Flachowsky, G. (2003) Safety assessment of Bt 176 maize in broiler nutrition: degradation of maize-DNA and its metabolic fate. *Tierernahr*, 57: p. 235–252.
- Tudisco R., Lombard i.P., Boera F., d'Angelo D., Cutrgelli M.I., Mastellone V., Terzi V., Avallone L., Infascelli F. (2006) Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis. *Anim. Sci*, 82: p. 193–199.
- Yonemochi C., Fujisaki H., Harada C., Kusama T., Hanazumi M. (2002) Evaluation of transgenic event CBH 351 (StarLink) corn in broiler chicks. *Anim. Sci*, 73: p. 221–228.