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ORIGINAL PAPER

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# The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material

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**Abstract** The fate of ingested recombinant plant DNA in farm animals (cattle and chicken) being fed a diet containing conventional maize or recombinant *Bacillus thuringiensis* toxin-maize (Bt-maize) is described. The probability of the detection by polymerase chain reaction of chloroplast-specific gene fragments of different lengths (199 bp and 532 bp) and a Bt-maize-specific fragment [truncated version of *Cry*IA(*b*)] is shown. First data indicated that only short DNA fragments (<200 bp) derived from plant chloroplasts could be detected in the blood lymphocytes of cows. In all other cattle organs investigated (muscle, liver, spleen, kidney) plant DNAs were not found, except for faint signals in milk. Furthermore, Bt-gene fragments possibly recording the uptake of recombinant maize, were not detected in any sample from cattle. However, in all chicken tissues (muscle, liver, spleen, kidney) the short maize chloroplast gene fragment was amplified. In contrast to this, no foreign plant DNA fragments were found in eggs. Bt-gene specific constructs originating from recombinant Bt-maize were not detectable in any of these poultry samples either.

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## Introduction

With the introduction of genetically modified plants to the European market during the last few years, a public discussion has arisen concerning the safety and advantage of food produced in this way. The novel food regulation of the EU (EC No. 258/97) sets out the special requirements for plant-derived food and a "substantial equivalence" has been proposed. Special control mechanisms are necessary to monitor any irregularities during food production. Within the last few years several approaches to detecting recombinant plant DNA in food for man e.g. that produced from Bt-maize [1, 2] have been published. Simultaneously, genetically modified plants such as maize and soya beans have and will be increasingly used for the feeding of farm animals. Some studies investigating the safety of such forage plants, mainly describing the substantial equivalence have been presented. However, no statistical differences have been reported between the recombinant and conventional types forage plants like maize and soya beans [3–6]. If a further evaluation of these secondary animal products derived from forage plants is wanted, objective scientific research data should accompany such processes. Possible nucleic acid transfers from these plants into human beings can be assumed, but the significance of this process is still unresolved. Farm animals ingest up to grams of nucleic acids via feed day per day – this is a well known principle since animals eat other DNA-containing organisms. From recent reports it seems possible that such foreign DNA-fragments could be integrated into cells of the immune systems of rodents [7, 8] and they were detected for several hours within various other organs. This makes it necessary to think about the fate of plant DNA in farm

animals, especially where recombinant feed plants are concerned.

The aim of this study was to evaluate the possible transfer of plant DNA into two different farm animal species (cattle and poultry) with a special emphasis on detecting recombinant *Bacillus thuringiensis* toxin-maize (Bt-maize) material in secondary animal products like meat, eggs or milk. Therefore, a quick and sensible polymerase chain reaction (PCR) assay was introduced to test for the presence of residual universal plant and Bt-maize genes in animal samples.

## Materails and methods

### Animals and diets

*Poultry.* Twelve male broilers (Lohmann Meat) and twelve female laying hens (LSL, Lohmann White) were caged under standard conditions and fed during the experimental period as indicated [9, 3] [broiler: 50% conventional Cesar maize hybrid or Bt-Cesar maize (Novartis), 5% wheat, 39% soya bean extract, 2.8% soya bean oil, 3.2% premix; hens: 50% conventional Cesar hybrid maize or Bt-Cesar maize, 18.7% wheat, 18.7% soya bean extract, 12.6% mineral-vitamin-amino acid-premix]. The daily feed uptake was 115 g. At the end of the period all chicken were slaughtered and several tissues (liver, spleen, muscle) were carefully prepared for DNA analysis and stored at –20 °C.

*Cattle.* Forty fattening cattle (Deutsche Holstein) were kept under standard conditions [5] from when they were 188 kg until they reached 550 kg weight (246 days). All animals were fed with either conventional Cesar or Bt-Cesar maize silage ad libitum. An average daily intake of 18.8 kg maize silage was observed. Tissues (blood, liver, muscle, spleen, kidney) were sampled immediately after slaughter and stored at –20 °C.

Four lactating, fistulated cows (Deutsche Holstein) were kept under comparable conditions and milk, blood, chyme from the small intestine and faeces samples harvested for subsequent DNA analysis. Additionally, 20 ml blood samples were collected from different cows (2×5 Swiss Braun), and the lymphocytes separated using Ficoll (Pharmacia, Freiburg, Germany) and selected for DNA extraction. All samples were stored at –20 °C until analysis. The preparation of all samples was carried out as carefully as possible to avoid any cross-contamination.

### DNA extraction and gene-specific PCR

Tissue samples (100–300 mg wet weight) from chicken or cattle, milk (100 ml), eggs (100–300 mg wet weight) and faeces (100–300 mg wet weight) were extracted for total DNA using two commercial kits based on a combination of guanidinium-isothiocyanate GTC and silica-based DNA-isolation (Roche, Mannheim, Germany; Macherey & Nagel, Düren, Germany). Resulting DNA was quantified by UV-spectroscopy and additionally a more sensitive fluorescence dye-interacting method (Bisbenzimide-method).

Different PCR amplifications for a ubiquitous plant chloroplast gene (plant1 and plant2) and the specific gene sequence to detect Bt-maize [truncated crystal protein gene *1A* (CryIA)-construct] were performed as follows.

*Chloroplast-specific primers (for all plants with slightly differing product sizes).*

Plant 1: universal plant chloroplast primers as described by [10] [a highly homologous part in the non-coding region between the *trnT* and *trnF* genes of the chloroplast DNA (cpDNA)].
Plant 1 forward: 5′-CGA AAT CGG TAG ACG CTA CG-3′
Plant 1 reverse: 5′-GGG GAT AGA GGG ACT TGA AC-3′
Product: 532 bp for maize

Plant 2: newly designed for this project from a large (532 bp) maize plant PCR sequence.
Plant 2 forward: 5′-GGA AGC TGT TCT AAC GAA TCG-3′
Plant 2 reverse: 5′-CTC GAA AAC AAT GAA TTG AAG G-3′
Product: 199 bp for maize and other plants

*Bt-maize-specific primer pair.*

According to [1]:
CryIA3 forward: 5′-CCG CAC CCT GAG CAG CAC-3′
CryIA4 reverse: 5′-GGT GGC ACG TTG TTG TTC TGA-3′
Product: 189 bp

Universal cycling conditions were used for all PCR reactions introduced on a Personal Cycler (Biometra, Göttingen, Germany or Perkin Elmer, Germany): 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 1 min. The final stage was 72 °C for 3 min. All resulting PCR products were separated on 1.5% agarose gels containing ethidium-bromide, documented under UV-illumination using a VideoDoc-System (Pharmacia, Freiburg, Germany) or isolated and sequenced commercially (TopLab, Munich, Germany or MWG-Biotech, Ebersberg, Germany). Digital images of the gels were stored, inverted and their quality enhanced using the computer program PhotoShop (Adobe, USA).

## Results and discussion

We decided to introduce for this study two quick commercial DNA extraction protocols which we have not used previously [11], to provide enough pure DNA useful for PCR. Previous data [12] and the results of this study demonstrated that both methods are suitable for isolating enough residual DNA to generate distinct PCR products from all types of animal and plant materials dealt with in this study (Figs. 1–6).

To test for the suitability and detection sensitivity of two plant-specific primer-pairs a PCR detection during the fermentation process was first performed. It was possible to amplify the chloroplast-specific gene in all silage samples even after 12 weeks of maize plant fermentation (Fig. 1). This finding is in accordance with a previous report [13]. However, only the smaller amplicon (199 bp; Fig. 1A) generated reliable results, the larger one (532 bp) did not produce consistent results or failed to detect plant-specific signals after a pro-

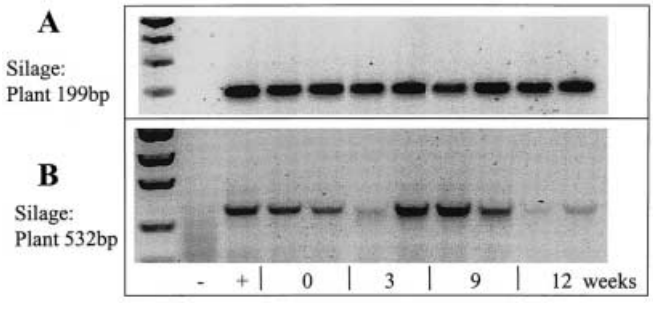


**Fig. 1A, B** Sensitivity of two polymerase chain reaction (PCR) assays detecting chloroplast plant DNA-fragments during the normal silage process of whole maize plants (increased maturation from fresh = 0 weeks until 12 weeks), amplifying **A** a shorter 199 bp PCR product or **B** a longer 532 bp PCR product. – Control without DNA; + positive control using maize standard DNA. Two out of five individual samples are depicted

longed silage process (Fig. 1B; 12 weeks). Therefore, due to this size-discriminating PCR system it could be assumed that the size of the DNA-fragments in silage decreased during the 12 week fermentation. To ensure that the smaller plant PCR amplicon could generate results when screening animal tissues a pilot experiment was performed with silage maize-fed cows. Since food material can reach the lymphocytes entering the intestinal wall directly, through Peyer's patches, we studied cows' peripheral blood lymphocytes: when using both plant-specific primers we got clear positive signals for the shorter 199 bp plant amplicon (Fig. 2A). As expected from the previous silage experiment the larger 532 bp plant PCR amplification failed in cow lymphocytes (Fig. 2B). Furthermore, the special construction and function of the ruminant gastrointestinal tract would limit further the uptake of complex plant DNA. This may indicate that any contaminations with whole plant DNA from exogenous sources can be excluded during that specific experiment.

In the following investigations only the small plant chloroplast primer-pair (plant 199 bp) was used to screen for plant DNA in cattle and chicken tissues. Additionally, a Bt-maize-specific primer pair (CryIA) was selected which amplifies a comparably small PCR product (189 bp) under similar cycle conditions.

## Searching for plant- and Bt-maize- specific DNA in cattle samples

When searching for amplifiable plant DNA fragments in cows fed with recombinant maize silage, the only positive signals which could be observed were in duodenal juice (chyme; Fig. 3A, B): both plant and Bt-maize-specific (CryIA) amplicons were present. In contrast, in blood and faeces no signals were detected. Occasional, very faint signals were detected for plant DNA in milk (Fig. 3A). Bt-specific gene material (Fig. 3B) has never been detected in any cow sample except for chyme.

As reported in a previous publication [14] PCR-amplifications followed by a lengthy southern blot detection using plant and 5-enolpyruvylshikimi-3-phosphate synthase (EPSPS)-specific (Basta-resistant soya bean) hybridisation probes never found a specific signal for EPSPS in cow lymphocytes or in milk after feeding cows recombinant soya beans. But plant DNA has been detected in lymphocytes using the Southern blotting technique, supporting our PCR results.

When all the fattening cattle samples were analysed no positive signal for any of the investigated tissues remained positive, for the plant or the *Cry*IA gene (Fig. 4A, B). The negative (–) and positive (+) controls verified that each PCR reaction did function properly.

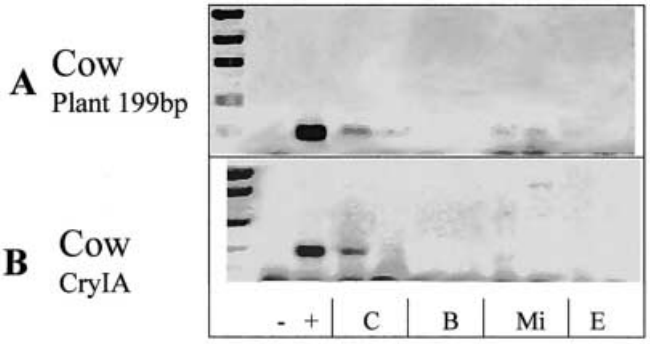


**Fig. 3A, B** PCR detection of DNA fragments in cow samples searching for **A** plant chloroplast or **B** Bt-maize (CryIA)-specific genes in various samples. – Control without DNA, + positive control, *C* chyme, *B* blood, *Mi* milk, *E* excrements. Shown are two typical examples out of five individual experiments

## Searching for plant- and Bt-maize- specific gene fragments in chicken samples

In all investigated broiler tissues, plant DNA fragments were successfully amplified (Fig. 5A): leg and breast muscles, liver, spleen and kidney were remarkably highly positive for the plant amplicon. In contrast, no Bt-maize-specific gene fragment was ever detected in these tissues (Fig. 5B). Visible background stainings on the gels did not represent *Cry*IA-specific signals (se-

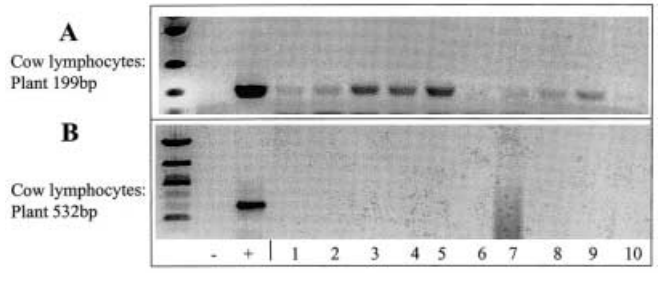


**Fig. 2A, B** Detection of chloroplast plant DNA fragments in blood lymphocytes from cows feed with maize silage. The **A** smaller 199 bp PCR amplicon was successfully amplified from blood lymphocyte DNA when compared to **B** the larger amplicon 532 bp. – Control without DNA, + positive control, 1–10 representing individual cow samples

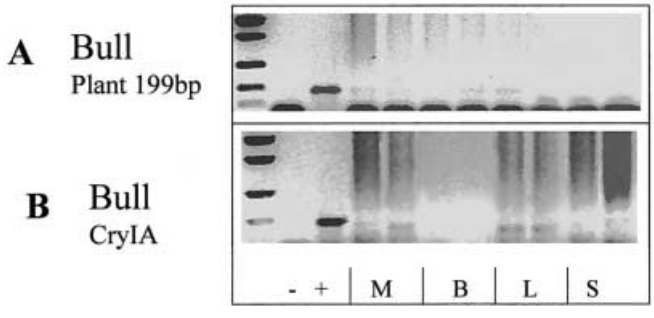


**Fig. 4A, B** PCR-detection of DNA fragments in bull samples searching for **A** plant chloroplast or **B** Bt-maize (CryIA)-specific genes in various samples. – control without DNA, + positive control, *M* muscle; *B* blood; *L* liver; *S* spleen. Shown are two typical examples out of five individual experiments

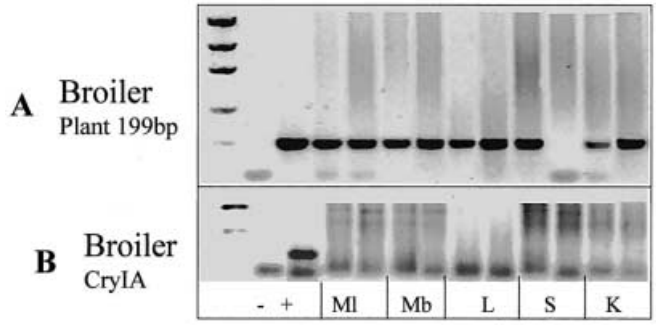


**Fig. 5A, B** Detection of **A** plant or **B** Bt-maize (CryIA) DNA fragments in broiler samples using specific PCR amplifications. – control without DNA, + positive control, *Ml* leg muscle; *Mb* breast muscle; *L* liver; *S* spleen; *K* kidney. Shown are two typical examples out of a series (17 for tissues) of independent experiments

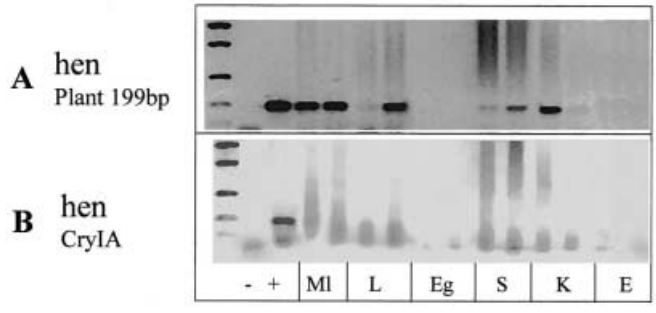


**Fig. 6A, B** Detection of **A** plant or **B** Bt-maize (CryIA) DNA fragments in hen samples using specific PCR amplifications. – Control without DNA, + positive control, *Ml* leg muscle, *L* liver; *Eg* egg; *S* spleen; *K* kidney; *E* excrements. Shown are two typical examples out of a series (17 for tissues, 5 for egg and excrements) of independent experiments

quencing of potential candidates always excluded them as specific amplicons).

Observing the hen samples similar pictures appeared: all organs (muscle, liver, spleen, kidney) prooved positive for plant DNA fragments, but no signals were detected in eggs or excrements (Fig. 6A). Similarly to the previous experiment, the Bt-maize-specific gene construct (CryIA) was not found in any of the hen samples (Fig. 6B). Two main reasons for the high incidence of detecting plant DNA fragments in all chicken organs may be (1) the application of unprocessed whole corn, and (2) the characteristics of the gastrointestinal tract of the chicken, including a very short digestion path. This may, when compared to the cattle experiments, explain the differences between results.

To ensure the specificity of the PCR amplification from the animal samples, some of the chloroplast plant PCR products (199 bp) extracted from cattle and chicken tissue were subsequently DNA-sequenced and compared with the known maize chloroplast sequence (European Molecular Biology Laboratory EMBL Acc.no. X86563) resulting in 100% homology.

In an attempt to further improve the sensitivity of the PCR detection method, different approaches were used in this study (silver staining after PAGE electrophoresis of PCR products, real-time PCR using the LightCycler technique, nested PCR, PCR-LCR), but none of these techniques were successful, mainly due to highly increased background signals. Therefore, these techniques have not been described here in detail.

In summary, this study leads to the assumption that forage plant DNA fragments are detectable in some farm animals if the amount of starting material is high enough (e.g. cpDNA) and the degradation stage of the DNA at the place of uptake (intestine) is not too high (such as following DNA fragmentation by silage and increased gastrointestinal storage time). Under such circumstances a concluding overview of our experiments can be drawn as follows (Table 1): (1) a minor transfer of small DNA fragments from highly abundant plant DNA (chloroplast DNA) can be observed exclusively into bovine lymphocytes and very few into the milk (near the detection limit); (2) in chicken organs the detection of chloroplast plant DNA was always successful, but not in eggs; and (3) relatively low levels of starting DNA material, as would be the case for the single Bt-maize gene construct within the huge maize genome (CryIA is approximately 50,000× smaller than the corresponding plant chloroplast gene fragment), led to the failure to detect specific CryIA fragments in all animal samples investigated in this study.

Throughout evolution, animals have been confronted with huge amounts of foreign DNA e.g. uptake through the gastrointestinal tract (up to grams per day), and such an uptake of high amounts of foreign gene material must be accepted as a normal event, for man and other animals [15]. Depending on the animal species and the type of food, a more or less significant transfer of foreign food DNA can be assumed. Various hypotheses have been proposed on how foreign DNA can reach the mammalian organism through gastrointestinal or placental portals, and which consequences should be considered [15]. It is of major interest that

**Table 1** Detection of plant chloroplast DNA (199 bp) by PCR in animal tissues. *n. d.* not determined, – no signals, *(+)* faint signal, + positive signal, ++ strong positive signal. Minimum sample number was $n=17$ for chicken, $n=5$ for eggs and excrements, $n=5$ for bulls and $n=4$ for cows

| | Cow | Bull | Chicken |
|---|---|---|---|
| Lymphocytes | + | n. d. | n. d. |
| Blood | – | – | n. d. |
| Muscle | n. d. | – | ++ |
| Liver | n. d. | – | + |
| Spleen | n. d. | – | + |
| Kidney | n. d. | n. d. | + |
| Milk | (+) | n. d. | n. d. |
| Egg | n. d. | n. d. | – |
| Chyme | + | n. d. | n. d. |
| Excrements | – | n. d. | – |

the fate of foreign DNA is not only degradation, but that a chromosomal integration and placental transfer may well be possible, as published recently [16, 17]. To our knowledge only rodents with a monogastric digestion system have been investigated; in contrast our study deals with two animal species (cattle and poultry) possessing differing gastrointestinal tracts. As shown, this can result in remarkable differences.

For the first time we reported here the uptake and fate of plant DNA in two farm animal species under normal feeding conditions. Therefore, it was not the case that a single pulse of foreign DNA was applied, but that a continuous administration of Bt-maize material over a longer period was realized. The chance of being able to detect DNA is limited by fermentation (silage) processes, mechanisms within the gastrointestinal tract and the turnover rate of DNA within the organisms. Therefore we decided to choose very short PCR amplicons to avoid poor sensitivity (the plant and Bt-maize primer pairs amplified a 199 bp and 189 bp fragment, respectively). Using such short amplicons increased the chance of detecting highly degraded and diluted DNA in complex samples as shown.

Possible problems of contamination during sample collection (e.g. farm plant material may be stored in the same room, and soya bean or maize dust can easily be transferred into milk) could be excluded during our sample collection due to separated areas. Additionally, we did not increase the PCR cycle number or use recycling, to minimise false positive signals. We would like to emphasis that our results are the very first attempt to diagnose DNA-uptake in farm animals; quantitative results cannot be expected from this approach and we are concerned that an approach that can produce such results will be very laborious to work out. If highly reliable quantitative techniques for detecting animal products fed with recombinant plants are desired, several facts must be taken into account: the precise selection of suitable PCR amplicons, the total DNA content and condition of the starting material (processed or not), the very small difference between transgenic and conventional genomes, that the recombinant construct must be previously known, the transfer and uptake into the deviating animals (ruminant, monogastric, digestion time etc.), and that the distribution of DNA-fragments within the animals is not even. Another problem will be the generation of more or less nonhomogeneous results; producing reliable, quantitative and significant data will not be easy using these techniques. But this will be absolutely essential for a later forensic examination.

Future developments will depend on acknowledging these results, in order to optimise the DNA extraction and detection sensitivity. However, this challenge should lead to new, more sensitive and cheaper technologies. But, to avoid introducing laborious and very complex protocols to screen for animal products produced from recombinant plants further research activities are necessary; a one-step-PCR will not be sufficiently sensitive and reliable for all requirements (vis difference between cattle versus chicken meat). The first steps towards detecting food products in animals fed with genetically modified plants have been taken, but there is much work to be done in order to possess reliable forensic methods that will work in the free market – today, the guaranteed success of a real forensic detection method remains questionable. During our collaborative case study the whole experimental procedure was difficult to perform in order to get consistent results out of it. Not all of the samples created the same signals in all laboratories – however, the main stream of all results was correct and was producing no false positive data, excluding any contamination. To stimulate future scientific work in this field we attempted to highlight some obvious problems which will be encountered in the search for the desired control mechanism of animal-derived genetically modified food. Today no safe indication for the biological relevance of highly degraded DNA can be provided to prove an interaction with the animals or consumers health – although it seems to be very unlikely that functional genes are transferred by this process. Possibly, long term experiments should be done to elucidate the influence of recombinant plants on the health of farm animals during years of feeding, and even humans might be included in such trials. Finally the cost – benefit correlation should have been considered before postulating the obligation of such analysis. If desired, easy testing for recombinant food plants directly on the farm will be the most successful and economic method of choice. This would support a voluntary monitoring system useful for farm and food cooperations interested in using a negative labelling of their products.

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