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Degradation and possible carry over of feed DNA monitored in pigs and poultry

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Abstract A possible carry over of foreign food DNA into the body after consumption was examined. After feeding pigs with conventional and recombinant (Bt-) maize, different body samples were investigated using DNA-extraction followed by PCR procedures to detect chloroplast genes of different length (199 bp and 532 bp), a maize-specific gene (zein) and a specific transgene present in Bt-maize (cryIa). Initially, a time-dependent degradation of feed DNA in the gastrointestinal tract of pigs was analysed within the juices from stomach and three parts of the small intestine (duodenum, jejunum, ileum). Subsequently, a possible transfer of residual chloroplast specific DNA as well as recombinant Bt-maize DNA fragments into different pig organs (blood, muscle, liver, spleen and lymph nodes) was examined. The suitability of the introduced DNA extraction procedure was verified through amplification of a universal gene (ubiquitin) demonstrating the successful PCR analysis within a range of 189–417 bp long DNA. Short chloroplast DNA fragments (199 bp) could be successfully amplified from the intestinal juices of pigs up to 12 h after the last feeding. In contrast, chloroplast-specific DNA was not found in any pig organ investigated so far. Specific gene fragments from the transgene maize (Bt-maize) were never detected in any pig sample.

A field study examining supermarket poultry samples (leg, breast and wing muscle, stomach) led to frequent detections of the short chloroplast DNA fragment (199 bp). Furthermore, faint signals for the maize specific zein gene fragment were detected in these poultry tissues. Additional PCR examinations using unhatched chicken embryos provided the first indication that neither chloroplast nor maize genes are present endogenously within the wild-type poultry genome. Therefore, a transient transfer of short forage DNA into most poultry organs can be suspected.

Keywords Feed-DNA · DNA transfer · *Bacillus thuringiensis* toxin maize · Polymerase chain reaction · Pigs and poultry

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Introduction

New regulations on genetically modified food and feed and possible labelling of according products are currently being discussed in the Commission of the European Union (information available from: http://www.transgen.de). In the first proposal, the labelling of feed that contains genetically modified plants is prescribed based on the possible detection of modified protein or DNA. With those regulations the legislator takes customer's concerns into account regarding, e.g. transfer of antibiotic resistance or allergenic properties of integrated genes as well as to be informed on production and contents of food [1, 2]. However, labelling of animal-derived products such as meat, milk or eggs is not provided. This coincides with the few available scientific data where recent publications emphasized the compositionally "substantial equivalence" for the main contents (protein, fat, fibre, ash, carbohydrates), amino acids and fatty acids of conventional soybeans and the glyphosate-tolerant soybeans [3]. Feeding studies that compare modified soybeans and modified maize with conventional products consistently showed no effects on the nutritional assessment of different animals such as rats, chickens, broiler chicken, catfish, dairy cattle, bulls or sheep [4, 5, 6, 7]. Only rare data are available on the fate of orally ingested DNA and a possible carry over. Most of the DNA seemed to be inactivated and degraded by the low pH in the stomach or nucleases produced in the saliva and the small intestines [8, 9]. Nevertheless in some cases small DNA fragments may pass through the gastrointestinal (GI) tract due to

binding onto soil minerals or proteins [10, 11] which could act as a protection against degradation. On the other hand, the incorporation of foreign DNA fragments into the body is described for rodents, supposable through Peyer's patches in the GI tract containing M-cells [12, 13, 14, 15, 16, 17]. In this regard for chicken the appearance of M-cells in the gut-associated lymph tissue has been recorded [18]. However, long term feeding of mice for eight generations did not indicate a germ line transfer of orally ingested foreign DNA [19]. The aim of this study was to monitor the time-dependent degradation of feed DNA in the porcine GI tract as well as to trace a questionnaire carry over of feed DNA into pig organs using DNA extraction and PCR methods. Furthermore, commercial available poultry samples were introduced for that analysis as a first field study continuing our former study [13]. A clarification of an endogenous genomic presence of homologous plant DNA was performed using chicken embryo directly from eggs.

Materials and methods

Animals and diets

Pigs (first experiment: fate of feed-DNA in organs). Thirty five male and female pigs (Pietrain×Deutsche Landrasse) were fattened from 29 kg at 70 days of age to 114 kg at 178 days of age with conventional dry feed mixture based on grain and proteins. Additionally, the animals received 25% maize in the beginning and 20% maize during the end of the mast. The control group (containing 18 animals) was fed with the conventional maize "Pactol". The examination group (containing 17 animals) was fed with the transgenic maize "Pactol Bt". Each pig received an average of 227.3 kg of conventional day feed mixture which corresponds with an overall intake of 50.2 kg of maize, a daily intake of 465 g maize/day per animal and an amount of 22.1% maize during the whole period. The pigs were kept sober for the last 18 h before slaughtering. The maize was harvested with a corn humidity of 30–35% and fed after drying. The conventional and the transgenic maize was stored separately to minimize possible cross contamination. After slaughtering samples of muscle, liver, spleen, lymph nodes and blood were taken and immediately stored at –20 °C. The sample preparation was carried out with cleaned instruments as carefully as possible to avoid potential contaminations between the different organs.

Pigs (second experiment: time-dependent degradation of DNA in the GI tract). Fourteen female pigs were fed with conventional maize until 36 h before slaughtering. Afterwards they were fed with one more ration of 1 kg feed with a content of 50% transgenic maize each at 12, 8, 6, 4 and 2 h (n=3 animals) before slaughtering. Blood samples were taken immediately after the death of the animals; stomach and small intestine (duodenum, jejunum and ileum) juices and intestine related lymph nodes were collected after the separation of the GI tract with clean instruments. All samples were stored and transported at –20 °C.

Chicken embryos. Five chicken hens (Leghorn) were fed with a standard breeding diet and the resulting five eggs were obtained containing seven days old chicken embryos. These eggs were carefully opened at the air bubble with a scalpel, the embryos separated with clean tweezers and DNA was extracted immediately from the whole corpus.

Poultry samples. Commercial poultry samples (turkey hen or chicken breast muscle, leg muscle, stomach and wings) were received from local supermarkets and stored at –20 °C until DNA was extracted. The feeding conditions for this field test could not be controlled in this experiment.

DNA extraction

All the tissue part samples to be extracted were cut out from the middle of the organ with a sterile individual scalpel blade to minimize contamination from the surface. DNA from pig's muscle, liver, spleen, lymph node, blood and juices from pig's duodenum, jejunum and ileum samples, chicken embryos and commercial poultry samples (100–300 mg wet weight each) was extracted with commercial kits using silica-columns (Roche, Mannheim, Germany). Quantity and purity of the obtained DNA was measured by OD 260/280 nm ratio and fluorescence dye technology on the LightCycler (SYBR Green I).

Gene-specific PCR

PCR amplification of Ubiquitin, chloroplast, Bt-maize or zein gene fragments. Block PCR reactions were performed on a Biometra cycler (Goettingen, Germany) with specific primers for Ubiquitin creating two PCR-products (189 bp and 417 bp) [20], a maize specific zein gene (277 bp) [21], two chloroplast PCRs that amplify products with different length respectively (plant 1=532 bp [22] and plant 2=199 bp [11]) and a PCR for Bt-maize specific truncated cryIa gene (211 bp) [23]. Additionally, the following new Bt176 primers were introduced resulting in a more robust PCR: Bt176.for: 5′-TTTTTTCCTCCCGATACGC-3′ and Bt176.rev: 5′-TCAGCAGGAACTGGGTCAG-3′. PCR product length was 251 bp.

Those Bt176 PCR primers were chosen using the "HUSAR" program package via Internet (http://genome.dkfz-heidelberg.de/menu/husar). Each PCR was performed using following conditions: an initial denaturation step at 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 3 min. A single reaction mixture contained 18.4 µl H_2O, 2.5 µl 10× buffer (Roche, Mannheim, Germany), 0.5 µl of each primer (20 µmol/l) and 0.1 µl Taq DNA-Polymerase (5 U/µl; Roche). As template 2.5 µl of a DNA solution in different dilutions was introduced.

PCR products generated from different positive samples were extracted and sequenced (Toplab, Martinsried, Germany). The nucleotide sequence received was compared with known fragments from databases and sequenced PCR products from the forage plants to confirm the authenticity of the PCR products.

Results and discussion

Fate of feed chloroplast and Bt-maize specific DNA in pig organs

Initial tests with both the conventional and the genetically modified fodder maize served as a control for detecting Bt-maize and chloroplast specific gene fragments. As expected, all feed DNA extracts resulted in a strong positive signal for the plant specific chloroplast genes. The PCR for the Bt-maize only showed strong positive signals for the genetically modified maize samples which indicates that a cross contamination of the conventional maize can be excluded (data not shown). With all DNA extracts from pig tissues and blood samples a Ubiquitin PCR verified the overall efficiency of the amplification procedure with product lengths of 189 bp and 417 bp respectively. Therefore, it can be supposed that all DNA

extracts used do not contain any inhibitors and PCR will be performed properly. Even so, all PCR reactions with the pig tissues and blood samples never generated any visible product neither for the highly enriched chloroplast-DNA nor for Bt-maize specific fragments (data not shown). These reactions were performed and cross-checked with different dilutions and pooled samples to verify the results. This fact makes the assumption of a DNA transfer through the epithelial cell layer of the GI tract into the blood stream for the porcine species very unlikely. This indicates obvious differences between the species when taking into account previous data published recently for cows and chicken [13].

Time-dependent detection of chloroplast specific DNA in GI tract of pigs

As described for the pig organs, an initial control PCR was performed using Ubiquitin primers for all stomach, duodenum, jejunum, ileum, blood and small intestine associated lymph node DNA samples, generating clear PCR products of 189 bp and 417 bp. Based on former results indicating the beneficial use of the plant2 PCR amplifying the smaller chloroplast gene fragment of 199 bp, only this analysis was carried out. Therefore, in extracted DNA from juices of stomach and the small intestine, those short chloroplast specific fragments could be well detected. For the stomach juice clear positive signals were still received after 6 h and faint signals even after 12 h. In duodenum juice chloroplast DNA could be detected until 8 h between last feeding and slaughtering and with faint signals after 12 h. Both in jejunum and ileum juice faint signals were received after 6–12 h and strong bands were detected up to 4 h after feeding. In accordance with the above-mentioned results for pig tissue samples the detection of chloroplast DNA fragments in blood or lymph nodes was not possible (Table 1). Additional negative and positive controls verified that each PCR reaction was functioning properly. These results clarify the expected time-dependent degradation of feed-

Table 1 Detection of short plant chloroplast DNA fragments (199 bp) using PCR in pig gastrointestinal tract samples (n=3). The hours indicate the time difference between last feeding and slaughtering

	Time				
	2 h	4 h	6 h	8 h	12 h
Stomach juice	+	+	+	(+)	(+)
Duodenum	+	+	+	+	(+)
Jejunum	+	(+)	(+)	(+)	(+)
Ileum	+	+	(+)	(+)	(+)
Blood	–	–	–	–	–
Lymph nodes	n.d.	–	–	–	–

n.d.=not determined
–=no signal
(+)=faint signal
+=positive signal

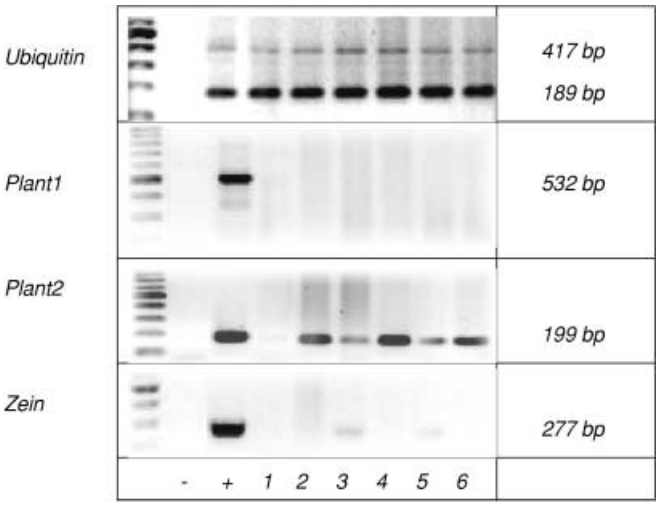

Fig. 1 Detection of different DNA fragments (Ubiquitin, Chloroplast DNA, zein) in supermarket poultry samples by PCR: –=negative control without DNA; +=positive control using chicken muscle DNA combined with maize standard DNA; 1=turkey hen breast muscle; 2=turkey hen liver; 3=chicken leg muscle; 4=chicken breast muscle; 5=chicken stomach; 6=chicken wings. One out of three samples is shown. Inverted image

DNA and the influence of a retention period in the GI tract of the examined pigs. Furthermore, a specific detection of Bt-maize specific cryIa fragments failed, possibly indicating a time- and mass-dependant degradation process of single genes. Hence, it could be shown that feed DNA appears in small fragments in the GI tract of pigs depending on the ingested amounts. The cryIa-gene that presents only a single copy in the genome of maize occurs much less often than the chloroplast fragment. This might be an explanation for the positive detection of chloroplast gene fragments and no hint for Bt-maize specific molecules. As described in prior publications [11, 12, 13, 14], it might be a rare event that single molecules could cross the epithelial cell layer in the small intestines. Under those circumstances it appears improbable having a significant transfer or survival of whole functional genes in the porcine GI tract containing more than 500 bp.

Detection of chloroplast specific DNA in supermarket poultry samples

For the DNA extracted from all commercial poultry samples, the control Ubiquitin PCR could be performed detecting DNA fragments representing a length between 189 bp and 417 bp (Fig. 1). Furthermore, the detection of short chloroplast fragments was successful for all field samples. Comparison of the amplification efficiency of the shorter (199 bp) vs the longer (532 bp) fragment showed a higher probability of detecting the short fragment. Additionally, the maize specific zein fragments could be detected with very faint signals in chicken leg muscle and chicken stomach (Fig. 1).

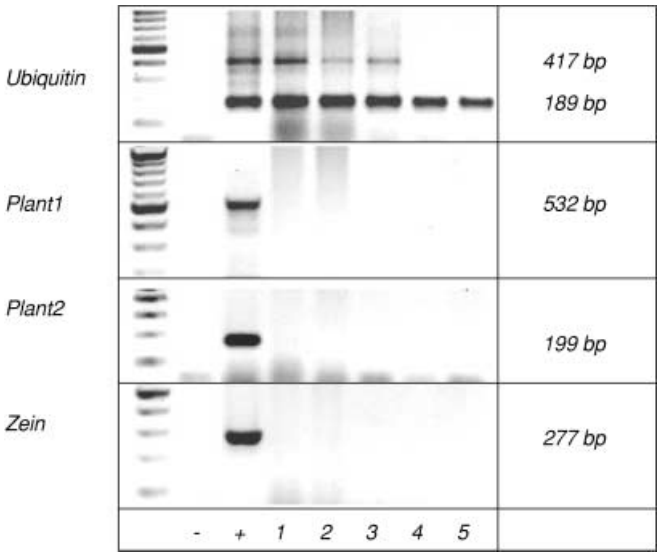

Fig. 2 Detection of different DNA fragments (Ubiquitin, Chloroplast, zein) in chicken embryo samples by PCR: –=negative control without DNA; +=positive control using chicken muscle DNA combined with maize standard DNA; 1–5=five individual chicken embryo DNA samples. Inverted image

Therefore, it can be considered that an incomplete degradation of ingested DNA fragments may take place in the GI tract of birds, enabling the detection of residual plant gene fragments. Due to a fast passage of feed through the GI tract of avians the appearance of DNA fragments might be more likely than for mammals. Therefore, the presentation of DNA molecules to the M-cells in the Peyer's patches of the intestine wall makes a transfer of such molecules into the birds' body probable. Even so, possible cross contamination from plant dust and due to improper slaughtering cannot be excluded for those samples. Although it is impossible to control the slaughter and subsequent sample collection, possible contamination during the sample preparation was reduced because DNA was extracted from the inner part of each tissue. On the background that such unspecific nucleic acid transfers are not unlikely this hypothesis has to be discussed in the context of resulting food contaminants.

Chicken embryos

Finally, in all chicken embryo DNA samples the endogenous Ubiquitin specific control PCR verified successfully the system performance (Fig. 2) indicating that no inhibitors are interacting. For further detection of plant DNA fragments three systems were used: two chloroplast DNA specific PCRs and a maize specific PCR (zein gene). For all chicken embryo samples none of these fragments could be amplified after maximum cycle numbers (Fig. 2). It clearly indicates that a germ line transfer of feed DNA in the case of avians can be excluded in contrast to rodents, where it was investigated but not proven [17]. The observed chicken embryos can be considered as animals not possessing any direct contact to feed plants. No homologous, endogenous plant or maize specific sequences were detected through PCR analysis. Putting together such results from embryos and commercial poultry samples, the assumption that chloroplast fragments in chicken muscles and organs are caused by ingested forage DNA from feed [11] but not by endogenous bird sequences can be supported.

In summary, all results coincide with former propositions about a possible transfer of small DNA fragments from feed into distinct farm animals. First data are now available for pigs, and a recent report first observing foreign DNA within various chicken organs is supported [11]. The diverse results found for different species may be caused by different digestion systems and therefore different length of presence of plant specific DNA in the GI tract. Additionally, different architecture of the colon and distinct transfer mechanisms through the lumen-blood barrier might be possible reasons of different transfer reactions. However, a detailed physiological research will be necessary for scientific clarification of the observed DNA transfers.

For the elaboration of further regulations it has to be considered if a more sensitive detection procedure would be desirable.

Whether future forensic methods are more powerful and reliable enough to enable the detection of specific residual DNA derived from feed or food in animal products is unknown. However, it will be difficult to control future regulations concerning labelling of meat from animals fed with genetically modified organisms, and has probably to be adjusted individually for each animal product.

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