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DETECTION OF PLANT SPECIES-SPECIFIC DNA (BARLEY AND SOYBEAN) IN BLOOD, MUSCLE TISSUE, ORGANS AND GASTROINTESTINAL CONTENTS OF RABBIT

Tudisco R., Calabrò S., Bovera F., Cutrignelli M.I., Nizza A., Piccolo V., Infascelli F.

Department of Scienze Zootecniche e Ispezione degli Alimenti, Faculty of Veterinary Medicine. University of Napoli Federico II. Via F. Delpino 1. 80137 Napoli, Italy.

Abstract: The aim of this study was to detect plant DNA sequences from low copy number genes of barley grain and soybean meal, the latter being subjected to solvent extraction process, in blood, liver, kidney, spleen, muscle tissue and digesta (duodenum, caecum and faeces from rectal ampulla) of rabbits. For fattening, Hyla rabbits (20 males and 20 females) were fed a diet including barley grain (15%) and soybean meal (12%). Animals were slaughtered at 74 d of age (2 ± 0.2 kg live weight) and samples collected from each animal. The quality of each DNA sample was verified using the UNIV P/Q primers used to amplify a mammalian specific portion of mtDNA 16S rRNA gene. The presence of plant DNA was subsequently ascertained on the same DNA samples, as well as on barley and soybean (control). Two classes of plant DNA sequences were monitored via real-time PCR, using SYBR® Green I Dye: a high copy number chloroplast gene (*trnI*) and a low copy number specific for barley (metal-dependent hydrolase-like protein) and soybean (lectin) genes. Melting curve analysis was used to identify the PCR products. The chloroplast fragment detection frequency was higher (P<0.01) in muscle (90%), liver (80%), kidney (80%) and spleen (80%) than in blood (40%) and digesta samples. In the latter, chloroplast DNA was found in 40 and 30% of duodenum and caecum contents respectively, and in 30% of faeces. The specificity of the amplicons obtained was checked by sequencing and annotation. In the samples positive for chloroplast fragments, the frequency of detection of barley specific sequence was higher (P<0.01) in liver (62.5%), kidney (62.5%), spleen (62.5%) and digesta (100%) than in blood (25%) and muscle (22.2%) samples. The soybean lectin gene was not detected in animal samples, although it was seen in plant samples. Results confirm that, except for gastrointestinal tract (GIT), plant single copy genes are more difficult to identify in animal samples.

Key Words: rabbit, soybean meal, barley grain, DNA fate, real time PCR.

INTRODUCTION

Transgenic crops are increasingly entering the feed market, and the fate and integrity of plant DNA in human and animal systems is a major public concern. Indeed, integrity of DNA is a key factor for hypothetical horizontal gene transfer of recombinant DNA from genetically modified (GM) crop-derived feeds to human and animal gut microflora (Netherwood *et al*., 2004).

The survivability of specific plant genes and proteins in animal systems has been evaluated by several studies, suggesting that absorption of plant DNA across the intestinal barrier is a natural event. Indeed, endogenous (native) plant genes have been detected in tissues and products from poultry (Aeschbacher *et al*., 2005), swine (Mazza *et al*., 2005) and ruminants (Nemeth *et al*., 2004).

Moreover, it is widely recognised that detectability of plant gene fragments is a function of copy number (Nemeth *et al*., 2004). Depending on the tissue type, a plant cell may contain 500-50000 copies of the

Correspondence: R. Tudisco, tudisco@unina.it
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chloroplast genome, in sharp contrast to the single copy gene in a nuclear genome (Benedich, 1987), so it should have a much higher probability of detection.

Digestibility of diets may also affect the persistence and, therefore, the detectability of feed ingested DNA. Intact plant cells confer protection to genetic material when feed passes through a number of hostile environments. However, severe feed-processing techniques will contribute to plant cell lysis and increase the release of larger quantities of DNA in the environment. Plant DNA detection is affected by both physical and chemical treatments, as well as by feed preservation methods (Forbes *et al*., 1998; Chiter *et al*., 2000). In our previous research (Tudisco *et al*., 2007) we demonstrated that the pelleting process negatively affected the detection of barley species specific plant DNA in blood, organs, muscle tissue and contents of the gastrointestinal tracts of rabbits.

The aim of this study was to detect plant DNA sequences from low copy number genes of barley grain and soybean meal, the latter being subjected to solvent extraction process, in blood, liver, kidney, spleen, muscle tissue and gastrointestinal contents from rabbit.

MATERIALS AND METHODS

Animal and diets

Forty weaned Hyla rabbits (20 males and 20 females) were fed *ad libitum* a diet (% as fed: 16.7 crude protein, 2.0 ether extract, 33.7 neutral detergent fibre) consisting of dehydrated alfalfa (40%), barley grain (15%), soybean meal (12%), sugar beet pulp (11%), wheat bran (20%). Water was provided *ad libitum*. The proximate composition of diet was determined according to AOAC 2000 (ether extract: 945.18; crude protein: 937.18; neutral detergent fibre: 2002.04). Rabbits were slaughtered at 74 d of age (live weight 2±0.2 kg).

Sampling of animal tissues and digesta

Immediately before slaughter, blood samples (~ 2 mL) were collected. Blood was withdrawn via auricular vein puncture. Before inserting the needle, the surface was cleaned and the first mL of blood discarded to avoid contamination. Blood samples were taken using a separate sterile needle and syringe for each rabbit and put in evacuated tubes containing K3-EDTA for DNA extraction. The tubes were kept in ice and stored at −20°C until the samples were processed.

After electric stunning, rabbits were slaughtered in an experimental slaughterhouse by cutting the carotid and jugular veins. Subsequently, 50 g of muscle tissue, 50 g of liver, one whole kidney and whole spleen as well as contents of the duodenum, caecum and rectal ampulla (faeces) were collected from each animal.

The organs and tissue (liver, kidney, spleen and muscle) were removed, with a clean scalpel blade, placed in clean nylon bags and then transferred to the laboratory where they were weighted. The contents of duodenum, caecum and rectal ampulla (faeces) of all rabbits were collected in plastic bags, placed on ice, and then transferred to the laboratory. Slaughter and sampling rooms were close but separate, to avoid possible contamination. During the sampling procedure special care was taken to avoid accidental contamination from the environment. The working place was accurately cleaned with a 5% hypochlorite solution rinsed in sterile water. Disposable gloves were changed between each organ sampling.

Sample preparation for DNA analysis

Feed samples. As control, a representative sample (~ 10 g each) of barley grain and soybean meal was prepared by finely grinding in liquid nitrogen using clean, sterile mortars and pestles. The ground material was then placed in labelled plastic tubes and stored at −20°C.

Tissue and digesta samples. About 2 g of muscle, liver, kidney and spleen samples were taken by cutting out an internal portion of the tissue with a clean scalpel blade in order to avoid possible contamination. These samples and a representative sub sample (about 2 g) of the fresh contents of the duodenum, caecum and rectal ampulla (faeces) of all rabbits were ground in liquid nitrogen. After grinding, each sample was divided into 3 aliquots (25 mg each for muscle and organs; 250 mg each for digesta) and immediately stored at −20°C in sterile tubes for DNA extraction.

DNA extraction methodologies and quantification.

Plant samples (100 mg) and duodenum and caecum contents (250 mg) were extracted according to the Wizard extraction method (Promega, Medison, Wis., USA). NucleoSpin® Tissue and NucleoSpin® Blood kit (Macherey-Nagel, Duren, Germany) were respectively used for extraction of tissue (25 mg) and blood (200 µL) samples according to the manufacturer's protocol. The DNA extraction methodologies are reported by Tudisco *et al*. (2006b). As for faeces samples, NucleoSpin® Tissue was slightly modified as follows: 1 mL TE buffer (10 mM Tris/Cl; 1 mM EDTA, pH 8) was added to 250 mg faeces and re-suspended by vigorous vortexing (30 s). After centrifugation at 4,000 *g* for 15 min, the pellet obtained was re-suspended in 800 µL lysis buffer. Two hundred µL of the re-suspended sample were transferred into a new microcentrifuge tube and incubated with 25 µL proteinase K solution at 58°C for at least 3 h on a shaking incubator. After digestion, the lysates from faeces were again incubated with 200 µL buffer B3 at 70°C for 10 min. Ethanol (210 µL, 96-100%) were added to the samples and the precipitate was loaded on the column placing into a 2 mL collecting tube and then centrifuged at 11,000 g for 1 min. The silica membrane was washed with 500 µL buffer BW and 600 µL buffer B5 following by centrifugation at 11,000 *g* for 1 min. After drying by centrifugation at 11,000 *g* for 1 min, the DNA was eluted with 100 µL pre-warmed elution buffer (70°C), incubated for 1 min, and the column centrifuged at 11,000 g for 1 min. Each sample was extracted in duplicate and stored at −20°C until used.

The DNA was quantified by spectrophotometer (Thermo Electron Corporation, Waltham, MA) and adjusted to 20 ng/µL prior to PCR. Then, its quality was checked from 260/280 nm UV absorption ratio and evaluated by separating 2 µL of each sample on a 0.8% (w/v) agarose gel in 1× TBE buffer (89mM Tris pH 8.4; 89mM boric acid; 2mM EDTA) and staining with ethidium bromide (10 mg/mL).

Oligonucleotide primers.

Four primer pairs (Table 1) were used to amplify specific DNA sequences of ~ 100 base pairs (bp). All DNA oligonucleotides were synthesised by Sigma-Genosys Ltd, Haverhill, United Kingdom, and diluted with an appropriate volume of sterilised ultrapure water to a final concentration of 100 pmol/µL, and stored at −20 °C until they were used. The UNIV P/Q primers were previously designed and assayed in real-time PCR by Sawyer *et al*. (2003) to amplify a region of the mtDNA 16S rRNA gene that is preserved among animal species. Clor 1/2 primers were designed on chloroplast *trnl* sequence and used to amplify DNA from several plant species (Terzi *et al*., 2004). Finally, in the samples positive for chloroplast gene, species specific primers for barley and soybean were used. ISC002F01 F/R primers were designed, using Primer Express software (Applied Biosystems, USA) on ISC002.F01F990411 EST (Expressed Sequence Tag) sequence (Gene Bank Accession BE411151), included in TC143986 by the TIGR Barley Gene Index (release 9.0, September 15, 2004) and tentatively annotated as a metal-dependent hydrolase-like protein. ISC002F01 F/R primers, after amplification of wheat, oat, barley, rice and maize DNA samples, give a barley-specific amplicon using melting curve analysis (data not shown). Le1n02 5/3 primers amplify the soybean lectin gene (Kuribara *et al*., 2002).

Table 1: Sequence, amplicon size (bp) and specific detection of primer pairs used in real time PCR for detection of animal and plant (multicopy and single copy) DNA.

Primer name	Sequenze (5'-3')	Amplicon size (bp)	Specific detection	References
UNIV P	GGTTTACGACCTCGATGTTG	104	mtDNA 16S rRNA (multicopy) gene	Sawyer *et al*. (2003)
UNIV Q	CCGGTCTGAACTCAGATCAC			
Clor 1	TTCCAGGGTTTCTCTGAATTTG	100	chloroplast (multicopy) gene, *trnl*	Terzi *et al*. (2004)
Clor 2	TATGGCGAAATCGGTAGACG			
Le1n02-5	GCCCTCTACTCCACCCCCA	118	soybean lectin (single copy) gene	Kuribara *et al*. (2002)
Le1n02-3	GCCCATCTGCAAGCCTTTTT			
ISC002F01 for	GGTGCACAGTGGTTAGTTGACAA	100	barley hydrolase-like protein (single copy) gene barley	the present study
ISC002F01 rev	AAGGCAACATGGGCAGTGAT			

Real-time PCR: SYBR Green detection.

Reactions for real-time PCR using SYBR Green detection consisted of 12.5 µL of SYBR® Green PCR Master Mix (Applied Biosystems, USA), 900 nM forward and reverse primers, DNA template (100 ng) and water to 25 µL. The PCR mixture was held at 50°C for 5 min and denatured at 95°C for 10 min. After this initial step, forty amplification cycles were carried out with the following conditions for each cycle: 95°C for 20 sec, 60°C for 1 min (for primers Clor 1/2, ISC002F01 F/R and Le1n02 5/3) or 55°C for 1 min (for primers UNIV P/Q). PCR reactions were performed on a 7300 Real-Time PCR System (Applied Biosystems, USA) and subjected to a heat dissociation protocol in the PE Biosystems 7300 software for melting (Tm) curve analysis. Following the final PCR cycle, the reactions were heat-denatured at 0.03°C/s over a 35°C temperature gradient from 60 to 95°C. All the reactions were done in duplicate.

After PCR amplification, Tm curve analysis were performed using the Dissociation Curve Analysis software (SDS v 1.2.3 System). Obtained fluorescence signals were continuously monitored during the slow warming-up gradient and showed a decreasing curve with a sharp fluorescence drop near the denaturation temperature. Plotting the negative derivate of the fluorescence over temperature versus the temperature (−dF/dT *vs*. T) generated peaks from which the Tm of the products was calculated.

In every PCR run, positive and negative controls were included in order to ensure reproducibility and absence of contaminants. For positive controls, reference DNA consisting of purified barley and soybean meal DNA was amplified in parallel with the samples to ensure the correct performance of the PCR; for negative control (NTC, buffer blank), water, instead of DNA, was added to the PCR mix to check for cross contamination with barley or soybean meal DNA in the PCR mix or its constituents (Klaften *et al*., 2004).

The PCR was done 3 times for each replicate and only samples showing positivity at least twice were judged as positive (Chowdhury *et al*., 2003).

PCR product sequencing and annotation.

The amplification products obtained with Clor 1/2 primers on rabbit DNA samples were separated on a 2.5% (w/v) agarose gel in 1×TBE buffer and the excised bands were purified following QIAquick gel extraction kit protocol (QIAGEN GmbH, Germany). Sequence reactions were performed using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and Clor 1 primer. Dye terminator excess was removed using the MultiScreen separation system (Millipore Corp., USA). Sequencing was performed using the ABI Prism model 310 capillary sequencer (Applied Biosystem, USA). Similarities with all sequences in the international nucleotide non redundant data banks and with sequences from EST division were detected using the BLAST program (Altschul *et al*., 1997) on network servers. Amplification and sequencing were done in duplicate.

Statistics

The presence of plant DNA fragments in blood, skeletal muscle, organs and digesta was analysed by using a Chi-square test (χ^2).

RESULTS

The quality of each DNA sample extracted from blood, tissue, organs and gut contents was verified using the UNIV P/Q primers, which were used to amplify a mammalin specific portion of mtDNA 16S rRNA gene.

The presence of plant high copy gene (chloroplast, *trnl*) was subsequently ascertained on the same DNA samples, as reported in Figure 1. Chloroplast DNA was found in 90% of muscle, 80% of liver, kidney and spleen, and in a lower (P<0.01) proportion in blood (40%) and digesta samples (40% in duodenum and 30% in caecum contents and faeces).

The specificity of the amplicons obtained was checked by sequencing and annotation. In Figure 2, we report the electrophoretic separation of PCR products obtained with Clor 1/2 primers on animal and plant

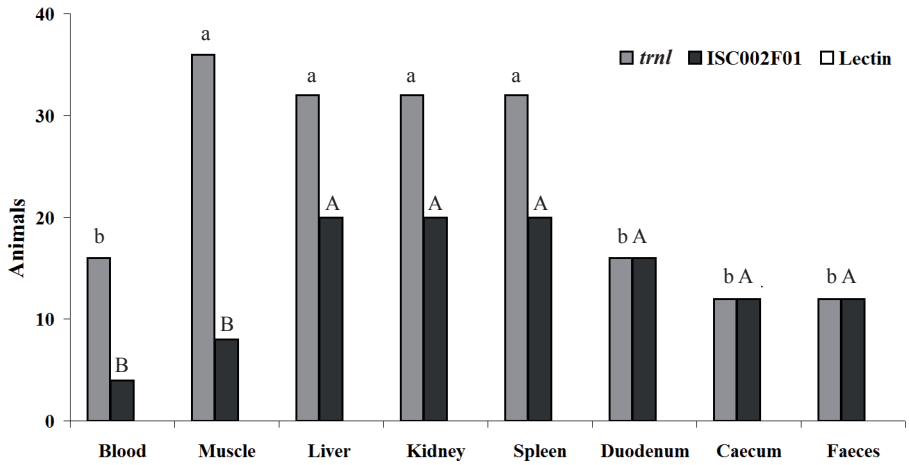
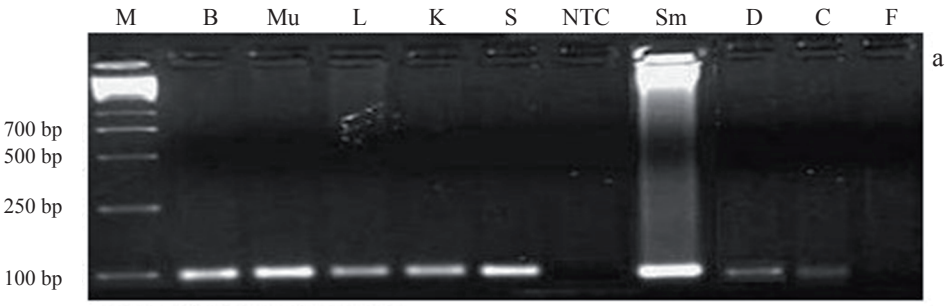


Figure 1: Detection of chloroplast gene (*trnl*) and species specific sequences (barley, ISC002F01; soybean, Lectin) by real time PCR in rabbit samples. Different letters (a, b) indicates values differing with P<0.01 *trnl* gene. Different letters (A, B) indicates values differing with P<0.01 for ISC002F01 gene. Lectin gene was not detected.



Gi2001194/gb AF519168.1 b

481 ttgcaataag caagcgaggt ctctcaaata gagtcgaact gaactgctag actacgttga
541 gtgatgaact caatgattca aaaaaaaaac taagagatgg atgaaattac acaaggaatc
601 ctggtctcaa agaagaggaa aatgggga**ta tggcgaaatc ggtagacgct acggacttga**
661 **ttgtattgag ccttggtatg gaaacctgct aagtggtaac ttccaaattc agagaaaccc**
721 **tggaa**ttaaa aaagggcaat cctgagccaa atccgtgttt tgagaaaaca aggggttctc
781 gaactagaat acaaaggaaa aggataggtg cagagactca atggaagctg ttctaacgaa

Figure 2: a) PCR amplification pattern of plant DNA fragments using Clor 1/2 primer pair on rabbit and plant sample. M: 1 kb DNA-marker, B: blood, Mu: muscle tissue, L: liver, K: kidney, S: spleen, NTC: no template controls, Sm: soybean meal, D: duodenum contents, C: caecum contents, F: faeces. The 100 bp bands amplified from animal samples were isolated from the gel and sequenced. b) The sequence of a tRNA Leu gene is reported, matching the sequences of the amplicons reported in (a). The locations of the homologous parts of the sequences are indicated with bold letters and the primers are underlined.

DNA samples, and the sequence obtained after purification of the amplified bands. The sequences were annotated after BLASTN analysis as plant *trnl* gene. All the sequences of PCR products obtained with Clor 1/2 primers on DNA samples gave the same result, confirming that we monitored a plant-specific sequence.

Concerning the barley specific fragment, in the samples positive for chloroplast fragments the amplifications were obtained in 25.0% of blood and 22.2% of muscle, whereas higher (P<0.01) values were obtained for the liver, kidney and spleen (62.5%) and 100% for duodenum, caecal contents and faeces. (Figure 1).

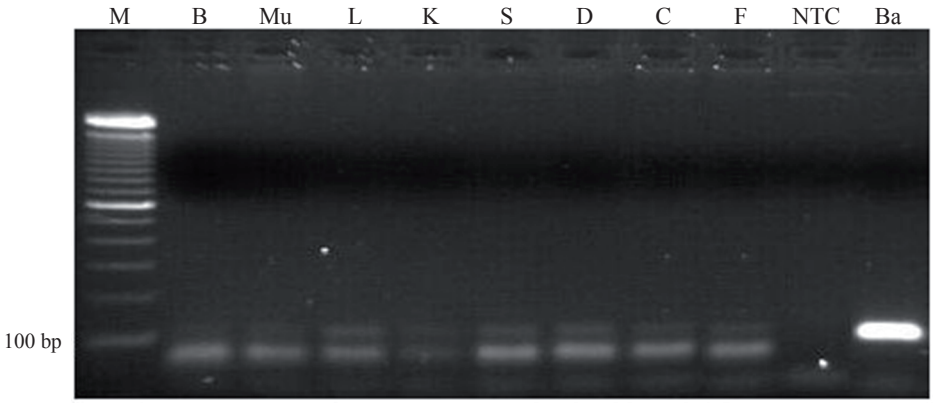


Figure 3: PCR amplification pattern of barley specific fragment using ISC002F01 F/R primers pair on rabbit and plant sample. M: 1 kb DNA-marker, B: blood, Mu: muscle tissue, L: liver, K: kidney, S: spleen, D: duodenum contents, C: caecum contents, F: faeces, NTC: no template controls, Ba: barley grain.

In contrast, the soybean specific fragments were not detected in animal DNA samples (Figure 1).

In Figure 3, we report the electrophoretic separation of PCR products obtained with ISC002F01 F/R primers on animal and plant DNA samples.

DISCUSSION

Our findings confirm the likelihood that plant DNA fragments can survive digestive processes (Einspanier *et al*., 2004; Duggan *et al*., 2003) and can be transferred to blood and organs. In this study, a higher incidence of feed DNA fragments was found in muscle and organs compared with blood and digesta samples, coinciding with previous results obtained in buffaloes (Tudisco *et al*., 2006a) and rabbits (Tudisco *et al*., 2006b). Moreover, Phipps *et al*. (2003) found chloroplast fragments in milk but rarely in blood from dairy cows. Concerning the gastrointestinal tract (GIT), plant DNA was detected in a similar proportion in the duodenum, caecum contents and faeces (Figure 1). However, previous studies showed evidence of degradation of free DNA in ruminant and monogastric GIT upon its release from partially or completely digested feeds (McAllan, 1980 and 1982; Flachowsky *et al*., 2005). In cattle, Einspanier *et al*. (2004) also found that ubiquitous plant chloroplast DNA fragments persisted more frequently in rumen and abomasal ingesta, compared to content from the lower GIT (colon).

In the samples positive for chloroplast fragments, the frequency of detection of barley specific sequence was higher ($P<0.01$) in liver (62.5%), kidney (62.5%), spleen (62.5%) and digesta (100%) than in blood (25%) and muscle (22.2%).

According to Artim *et al*. (2001), chloroplast DNA is easily recovered in animal tissues as a consequence of the larger quantity of copy number of a plastid gene than species specific gene, which is a single copy gene.

Barley specific gene was detected in 100% of GIT contents, probably because of the different structural composition of samples (organs and tissue *vs*. GIT contents).

Even though we did not compare processed with unprocessed soybean, our negative results in detecting lectin gene might be due to the solvent extraction. Indeed, according to Forbes *et al*. (1998) and Chiter *et al*. (2000), the feed processes using heat treatment can cause fragmentation of feed DNA. Therefore, a DNA fragmentation occurring during the process of oil extraction probably determines instability of soybean genome. This hypothesis is consistent with the results of Chen *et al*. (2005), who found that soybean processing caused DNA degradation. According to these authors, choosing primers for amplifying suitable DNA fragments can avoid false negative results in the testing and characterisation of processed food products.

Overall, our data add some information on the fate of feed plant DNA in animals, given that the persistence of DNA after dietary exposure is one aspect of risk assessment for new foodstuffs. Indeed, as concerns the hypothetical horizontal gene transfer of recombinant DNA from GM crop-derived feeds to animal and human gut microflora, Netherwood *et al*. (2004) found that a small proportion of feed DNA survives the transit through the human upper gastrointestinal tract and a very small proportion of the small intestinal microflora contains transgenic feed. According to the authors, even if this result does not indicate a complete transgenic transfer to the prokaryotes, the survival of transgenic DNA during the passage through the small intestine should be considered in future safety assessments of GM foods.

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