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Sensitive PCR Analysis of Animal Tissue Samples for Fragments of Endogenous and Transgenic Plant DNA

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An optimized DNA extraction protocol for animal tissues coupled with sensitive PCR methods was used to determine whether trace levels of feed-derived DNA fragments, plant and/or transgenic, are detectable in animal tissue samples including dairy milk and samples of muscle (meat) from chickens, swine, and beef steers. Assays were developed to detect DNA fragments of both the high copy number chloroplast-encoded maize rubisco gene (*rbcL*) and single copy nuclear-encoded transgenic elements (*p35S* and a MON 810-specific gene fragment). The specificities of the two *rbcL* PCR assays and two transgenic DNA PCR assays were established by testing against a range of conventional plant species and genetically modified maize crops. The sensitivities of the two *rbcL* PCR assays (resulting in 173 and 500 bp amplicons) were similar, detecting as little as 0.08 and 0.02 genomic equivalents, respectively. The sensitivities of the *p35S* and MON 810 PCR assays were approximately 5 and 10 genomic equivalents for 123 bp and 149 bp amplicons, respectively, which were considerably less than the sensitivity of the *rbcL* assays in terms of plant cell equivalents, but approximately similar when the higher numbers of copies of the chloroplast genome per cell are taken into account. The 173 bp *rbcL* assay detected the target plant chloroplast DNA fragment in 5%, 15%, and 53% of the muscle samples from beef steers, broiler chickens, and swine, respectively, and in 86% of the milk samples from dairy cows. Reanalysis of new aliquots of 31 of the pork samples that were positive in the 173 bp *rbcL* PCR showed that 58% of these samples were reproducibly positive in this same PCR assay. The 500 bp *rbcL* assay detected DNA fragments in 43% of the swine muscle samples and 79% of the milk samples. By comparison, no statistically significant detections of transgenic DNA fragments by the *p35S* PCR assay occurred with any of these animal tissue samples.

KEYWORDS: Biotechnology; DNA; maize; PCR; transgenic feed

INTRODUCTION

Modern plant biotechnology applies cellular and molecular biology techniques to transfer DNA into the germline of a crop, providing plant breeders with access to an array of traits for cultivars with new characteristics. These crops are variously termed biotechnology-derived, transgenic, or genetically modified (GM). Maize is a major source of animal nutrition with more than 60% of global maize production (80% of US-produced maize) being used as animal feed (*1*). In the U.S. in 2003, 40% of the maize planted was GM cultivars (*2*), and worldwide in 2002, 21% of the maize cultivars planted were GM (*3*). DNA, a component of all living plant cells, is present in plant products fed to animals and is digested along with other dietary components after ingestion. Questions about the digestive fate of transgenic DNA present in GM crops have been raised, especially in regard to whether fragments are detectable in products from animals fed those crops (*4*).

The application of sensitive molecular detection techniques using Polymerase Chain Reaction (PCR) methods can address the question of whether plant-derived DNA fragments are detectable in animal tissues. Some of these PCR studies have been recently reviewed (*5*−*7*). Without exception, published studies have shown that fragments of single copy plant transgenes were not detectable in tissues from animals fed GM crops. Of interest, however, are some reports describing detection of DNA fragments of abundant chloroplast-encoded genes in lymphocytes of dairy cows (*8*), in milk of dairy cows and in muscle, liver, spleen, and kidney of chickens (*9*), and in all parts of the gastrointestinal tract up to 12 h after feeding

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Table 1. Number of Samples Collected and Extracted for DNA from Various Species

sample type	MON 810 fed (no.)[a]	control fed (no.)[a]	ref
pork loin muscle	59	59	*22*
beef brisket muscle	10	10	*23*
chicken breast muscle	10	10	*24*
dairy milk	7	7	*25*

[a] 100% of the corn in the diet was the indicated type of corn grain under study.

pigs (*10*) using conventional PCR methods. A recent study of samples collected from throughout the digestive tract of swine (*11*) showed frequent detection of fragments of both transgenic (*Bacillus thuringiensis*, Bt, 211 bp) and an endogenous maize chloroplast gene ($tRNA_{leu}$, 532 bp) for up to 24 h after feeding (*11*). This report showed that the 211 bp fragment of the *Bt* gene was found in only a few digesta samples 48 h after feeding, with no samples containing detectable DNA fragments by 72 h. Also, none of the 324 swine tissue samples collected from nine tissues and taken throughout the 72 h period after feeding showed detectable fragments of transgenic DNA. In contrast, this study showed that a 140 bp fragment of the high copy number plant *rbcL* gene was detectable in several liver, ovary, and muscle tissue samples. These positive detections of chloroplast DNA fragments are possibly due to the 500−50000 copies (root versus leaves, respectively) of the chloroplast genome (*12*) relative to the single copy per haploid genome of a genomic transgene DNA target.

The present study was conducted to obtain additional data as to whether fragments of plant DNA are detectable in animal products, and to corroborate the observations that high copy number chloroplast plant DNA fragments are more detectable than fragments of single copy nuclear-encoded transgenes. Important to the present studies was the development of appropriate procedures that included proper controls to avoid false-positive and false-negative results, safeguards to avoid bias, such as analysis of samples in a double-blind manner, and application of PCR and DNA extraction methods optimized for sensitivity.

MATERIALS AND METHODS

Sample Collection and Processing. Samples for this experiment were collected from four independent animal performance studies, each conducted with diets containing MON 810 hybrid maize. In each of those animal studies, nontransgenic maize was fed to control animals, and samples were harvested as control material. The total number of samples collected and analyzed by species is listed in **Table 1**. The pork, beef, and poultry muscle samples were collected at the time of slaughter, bagged, and immediately placed on crushed dry ice prior to storage at −80 °C until subsampled. Milk samples were collected into sterile tubes using aseptic techniques and quickly frozen on crushed dry ice prior to storage at −80 °C until subsampled. All samples were shipped on dry ice to a laboratory for subsampling where no PCR products or plasmids had previously been handled (*13*). The frozen muscle samples were cut into subsamples weighing about 1 g (wet weight), ensuring that all external surfaces of the muscle samples were excised prior to collecting subsamples. Samples were kept on dry ice during the subsampling procedure. All tissue subsamples were placed in sterile polypropylene tubes pretreated with UV light and stored at −20 °C until analysis. Before DNA analysis, samples were assigned new sample IDs resulting in a double-blind sequence of samples. The Clinical Core Laboratory at Washington University, St. Louis, retained the key to the double-blind samples as an independent third party until PCR analysis was completed and they could match the results to the sample code.

DNA Extraction. All samples were extracted in duplicate. Each set of extractions was monitored for possible contamination by at least two extraction negative controls (buffer only). The data set was accepted only when the negative extraction controls were negative by PCR. If a positive PCR result was obtained with one or more extraction-negative control(s), the extraction of the whole set was repeated. Frozen muscle subsamples from chicken, pork, and beef (~1 g) were thoroughly homogenized with a mortar and pestle and incubated overnight at 60 °C under agitation with 7.5 mL of CTAB buffer [1.4 M NaCl, 2% (w/v) CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris, 15 mM EDTA, pH 8.0] and 30 μL of Proteinase K (20 mg/mL). Milk samples (2.5 mL) were directly mixed with CTAB buffer and Proteinase K and incubated as described above. Samples were then digested with 25 μL of RNase A (100 mg/mL) for 30 min at 60 °C under agitation. After centrifugation for 5 min at 3000*g*, the supernatants were used for DNA extraction.

For the chloroform extraction method, 900 μL of supernatant was extracted with 600 μL of chloroform. The aqueous phase was recovered, and DNA precipitated with 0.8 vol of 2-propanol and 1 μL of glycogen (20 mg/mL). Subsequently, the pellet was washed with 75% (v/v) ethanol and dissolved in 100 μL of 0.2X TE (2 mM Tris-HCl, 0.2 mM EDTA, pH 8.0). For milk, the whole supernatant was extracted with 5 mL of chloroform, and the subsequent steps were performed as described above.

For the CTAB precipitation method, 2 vol of CTAB precipitation buffer (40 mM NaCl, 0.5% (w/v) CTAB) were added to 600 μL of supernatant, mixed thoroughly, and incubated for approximately 60 min at room temperature prior to centrifugation for 5 min at 20000*g*. The pellet was dissolved in 350 μL of 1.2 M NaCl and then mixed with 350 μL of chloroform. The aqueous phase was recovered, and DNA precipitated with 1 vol of 2-propanol and 1 μL of glycogen. Subsequently, the pellet was washed with 75% (v/v) ethanol and dissolved in 100 μL of 0.2× TE.

For the silica gel purification method, the DNeasy Plant Mini Kit (Qiagen) was used according to manufacturer's instructions with slight modifications.

Aliquots of each DNA sample were loaded on a 1.5% (w/v) agarose gel, and the DNA yield was estimated by comparison with known amounts of high molecular weight calf thymus DNA.

MON 810 reference DNA was isolated from a single kernel of MON 810 maize according to the chloroform extraction method for milk described above but with the addition of a phenol extraction step before chloroform extraction. After measurement of the DNA concentration by UV spectroscopy, the relative copy number was calculated assuming a haploid genome weight of 2.6 pg for maize (*14*).

PCR Methodology. Two methods that resulted in different amplicon sizes were developed for the detection of the maize chloroplast gene encoding the large subunit of ribulose bisphosphate carboxylase (*rbcL*; GenBank Accession No. Z11973). The reverse primer (rbcl-r1: 5′-TGGTATCCATCGCTTTGAAACCA-3′) for both methods was derived from the 3′-end of the gene that is maize-specific due to a 3 bp insertion. In combination with two different forward primers (shown below), the rbcl-r1 reverse primer gave rise to 173 and 500 bp amplicons:

rbcl-f1: 5′-AGCTAATCGTGTGGCTTTAGAAGCC-3′ (173 bp product)

rbcl-f2: 5′-GCATTTCCGTGTATTAGCTAAAGCATTG-3′ (500 bp product)

Two methods were characterized for the detection of transgenic DNA fragments. The primers for these two PCR methods gave rise to a 123 bp amplicon [*p35S-PCR*; (*15*)] and a 149 bp amplicon [*MON 810-specific PCR*; (*16*)], as indicated next.

p35S PCR primers (123 bp product)

p35S-cf3: 5′-ccacgtcttcaaagcaagtgg-3

p35S-cr4: 5′-tcctctccaaatgaaatgaacttcc-3

MON 810-specific PCR primers (149 bp product)

mg3: 5′-actatccttcgcaagacccttcctc-3

mg4: 5′-gcattcagagaaacgtggcagtaac-3

DNA amplification was carried out in a final volume of 25 μL with 1× AmpliTaq Gold buffer (Applied Biosystems), 160 μM of each dNTP, 0.6 μM of each Primer, 0.8 U of AmpliTaq Gold Polymerase

(Applied Biosystems), and 5 μL of DNA. The cycling conditions on a GeneAmp 9700 thermal cycler (Applied Biosystems) were as follows: 10 min at 95 °C for initial denaturation, 50 cycles of amplification (25 s at 95 °C, 30 s at 62 °C, and 45 s at 72 °C), and final extension for 7 min at 72 °C. Aliquots of each PCR were examined on a 2.5% (w/v) agarose gel along with a suitable DNA marker.

The extracted DNA samples, including the extraction negative controls, were tested in the different PCR systems. To exclude false negative PCR results due to inhibition, spiked PCR reactions were set up in parallel to the analytical PCR whereby each sample extract was spiked with a fixed amount of positive DNA which was twice the number of target copies that were reproducibly detected during characterization of the PCR systems (see Results and Discussion). If a spiked reaction showed inhibition, both the analytical and the spiked PCR were repeated with both diluted and undiluted sample DNA. Alternatively, an additional purification step of the DNA was performed using a MicroSpin SH-300 column (Pharmacia) according to the manufacturer's instructions. However, if a sample was positive in the analytical reaction but the spiked reaction failed, it was scored positive without a repeat analysis.

Sequence Analyses. To confirm the sequence of the PCR amplicons, some reactions were separated by agarose gel electrophoresis, and the amplification products were excised and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Direct sequencing of the purified PCR products was performed at GATC Biotech AG, Konstanz, Germany.

RESULTS AND DISCUSSION

Optimization of DNA Purification from Animal Tissues. Identifying an efficient DNA extraction method that provides the maximum amount of input DNA per PCR is critical to the present studies of whether feed-derived plant and/or transgenic DNA fragments are detectable in muscle and milk samples. It is reasonable to expect that the vast majority of DNA extracted from animal tissue samples will be of animal origin, with only a small percentage of total extracted DNA having the possibility of being from dietary origin. Thus, efficient extraction methods are needed, and the methods have to yield very pure DNA with a minimum amount of PCR inhibitors. Three different DNA extraction methods (silica gel purification, CTAB precipitation, and chloroform extraction) were compared for muscle and milk samples. The same sample homogenization and lysis method was used in all cases.

Estimations of DNA yield by agarose gel electrophoresis showed that the best results were obtained using the chloroform extraction method (data not shown). Using this method, the DNA yield was in the range of ~20 μg per 100 mg of muscle tissue. However, none of the extraction methods produced visible amounts of DNA from milk as determined by agarose gel electrophoresis. For this reason, the two methods most amenable to being scaled up (CTAB precipitation and chloroform extraction) were adapted to process approximately 10-fold larger volumes of milk and retested. Using these methods, DNA extracted with both methods was visible by agarose gel electrophoresis. Considerably higher yields in the range of approximately 2 μg per mL of milk were obtained with the chloroform extraction method, although the milk yields were more variable from sample to sample than those for muscle. The quality of the extracted DNA and its suitability for PCR were assessed by testing the undiluted sample DNA in spiked *p35S* reactions. No appreciable PCR inhibition was observed for any of the DNA extracts even when relatively large amounts of genomic DNA (~1 μg of muscle-derived or ~0.2 μg of milk-derived DNA) were used in the PCR, indicating that the isolated DNA was of high purity and lacked noteworthy inhibitors.

Characterization of PCR Performance. To adequately address the primary objective of this study, it was important to establish PCR methods with optimized sensitivity for trace levels of plant DNA fragments in animal tissue homogenates. A number of key assay parameters, including specificity, sensitivity, and detection limit, needed to be determined for each PCR assay to ensure interpretable results.

Two PCR assays were developed for the chloroplast-encoded large subunit gene for maize ribulose bisphosphate carboxylase (*rbcL*). One PCR assay tested for a 173 bp fragment of the coding region of the *rbcL* gene, while the second PCR assay tested for a 500 bp fragment. To evaluate assay specificity of these *rbcL* PCR assays, genomic DNA from several plant species (potato, wheat, soy, sorghum, and rice) and one animal source (calf thymus) were investigated. It was found that both *rbcL* primer sets were specific for maize because they both produced their respective PCR amplicons only when DNA from maize was added to the PCR (data not shown). However, as *rbcL* gene sequences are ubiquitous throughout photosynthetic bacteria, fungi, and algae, the possibility of amplifying an *rbcL* DNA fragment present in a sample due to microbial origin cannot be completely ruled out.

As shown in **Figure 1**, an estimate of the detection limit (a measure of assay sensitivity) for both *rbcL* PCR assays was obtained by analyzing duplicate serial dilutions of MON 810 maize genomic DNA. To facilitate comparison of the detection limit for the different PCR methods, the amount of DNA per PCR is shown in **Figure 1** as the number of haploid genomic copies of MON 810 ("genome equivalents" or GE). It should be noted, however, that because each plant cell contains between 500 and 50000 copies of the chloroplast genome depending upon the plant tissue, the actual target copy number of chloroplast genomes per reaction would exceed the indicated value by a comparable factor (*12*). For this and all subsequent PCR experiments, observation of visible PCR products of the expected size with the duplicate samples was scored as a "positive" result, the lack of visible specific amplicons with both duplicates was scored a "negative" result, and if only one of the two duplicates yielded a visible product, it was scored an "indeterminate" result. The 173 bp PCR is capable of detecting the *rbcL* gene in a quantity of DNA equivalent to 0.02 GE. Indeterminate results were obtained at 0.005 GE. The 500 bp *rbcL* assay was slightly less sensitive at 0.08 GE. Indeterminate results were obtained at 0.02 and 0.005 GE. Thus, the sensitivity of the 173 bp and 500 bp assays appears to be similar.

The *cry1Ab* transgene in MON 810 maize is under the control of the CaMV 35S promoter and includes a part of the *hsp70* (heat shock protein 70) intron. An element-specific PCR system for the CaMV 35S promoter ("*p35S* PCR") was chosen for screening the animal samples for the single copy transgene. In addition, samples with a positive or indeterminate *p35S* result were also tested with a MON 810 construct-specific PCR method that detects the combination of the CaMV 35S promoter and the *hsp70* intron ("MON 810 PCR").

The *p35S* PCR assay produced the expected 123 bp amplification product with genomic DNA isolated from a range of GM maize products known to contain the *p35S* element (e.g., MON 810, Bt11, Bt176, Bt-Xtra, and StarLink) but not with DNA from Roundup Ready maize event GA21 that lacks the *p35S* element (data not shown). By comparison, the MON 810 PCR produced the expected 149 bp amplicon only with genomic DNA from MON 810 maize. Furthermore, neither PCR assay produced specific amplification bands when DNA from soy, brassica, wheat, rice, or nontransgenic maize was present, or using DNA from several animal species including chicken, pork, and cattle (data not shown).

The sensitivity of both the *p35S* and the MON 810 PCR assays was evaluated using multiple serial dilutions of MON

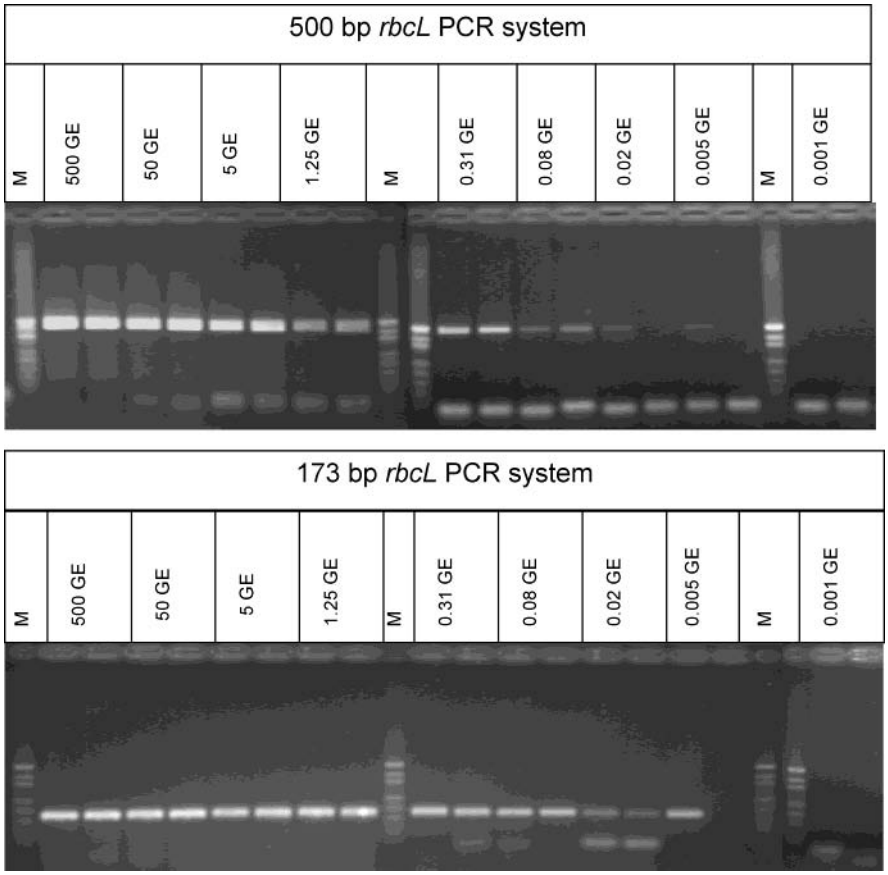

Figure 1. Sensitivity test of the 500 bp *rbcL* PCR system as compared to the 173 bp *rbcL* PCR system. Serial dilutions of MON 810 DNA were tested in both *rbcL* PCR systems. To compare the sensitivity results with other PCR systems, the amount of DNA is depicted in genome equivalents (GE) per reaction, which corresponds to haploid genomic copies of MON 810 DNA. M (DNA molecular weight marker): 501/498, 409, 325, 242, 190, 147, 110, and 67 bp.

810 maize genomic DNA (**Figure 2**). Plant DNA samples were tested in duplicate at concentrations ranging from 50 to 1 GE of MON 810 DNA per reaction. As with the *rbcL* assays, observation of visible PCR products of the expected size with the duplicate samples was scored as a "positive" result, the lack of visible specific amplicons with both duplicates was scored a "negative" result, and if only one of the two duplicates yielded a visible product, it was scored an "indeterminate" result. The *p35S* PCR gave a positive result with 5 GE of MON 810 DNA and an indeterminate result with 1 GE of MON 810 DNA. By comparison, the MON 810 PCR was less sensitive, showing positive results only with 10 GE, and indeterminate results with 5 and 2.5 GE. Thus, the detection limits for both the *p35S* and the MON 810 PCR methods were 5 and 10 GE, respectively.

Determination of Error Rates for the MON 810 PCR. To better understand the false positive and false negative error rates with sensitive PCR analyses such as those used in the present studies, the MON 810 PCR method was chosen as a representative test case. To assess the rate of false positive results with the MON 810 PCR method, 300 reactions were performed in the presence of background, known negative, porcine DNA (about 800 ng/reaction). For this purpose, 15 individual porcine extractions were performed, and each extraction was tested in 20 replicates using the MON 810 PCR method. No amplification bands were produced in 93% (278) of these 300 reactions. Amplification products in the size range of 90 to 500 bp were observed in 22 of the reactions (7%). Closer examination of the PCR results with these 22 runs revealed that none of the amplification products was a legitimate positive of the correct size of 149 bp. Consequently, the error rate for false positive results with the MON 810 PCR method was calculated to be $<1\%$ at the 95% confidence level as estimated by the proportion associated with a binomial distribution (*17*).

To evaluate the rate of false negative results with the MON 810 PCR method, 300 reactions were each spiked with approximately 10 GE of MON 810 DNA (26 pg) and assessed in the presence of background porcine DNA (about 800 ng/reaction). In approximately 97% (290) of these 300 reactions, the expected 149 bp PCR amplified product was produced, with only 10 reactions failing to yield an amplified product. Thus, at this very low spiking level of approximately 10 GE of MON 810 DNA per reaction, the rate of false negatives was approximately 3%. The limit of detection (LOD) of an assay is defined as the measured level of analyte at which a true positive sample gives a positive result 95% of the time. Therefore, this 3% false negative rate supports the conclusion that the estimated LOD of the PCR is close to the true LOD of the method.

Analysis of Animal Tissue Samples. Due to the high sensitivity of the PCR assays used for this study, all analyses were carried out using extensive precautions to avoid any laboratory-derived sample contamination during tissue handling, DNA extraction, and PCR. DNA extractions were carried out in duplicate. Two negative (buffer only) control extractions accompanied each set of DNA extractions to monitor for any contamination that could possibly have occurred during the DNA extraction procedure. If a positive PCR result was obtained with one or more extraction-negative control(s), the extraction of the whole set was repeated. In addition to these DNA extraction negative controls, all PCR analyses also included negative (buffer only) controls. To exclude false negative results

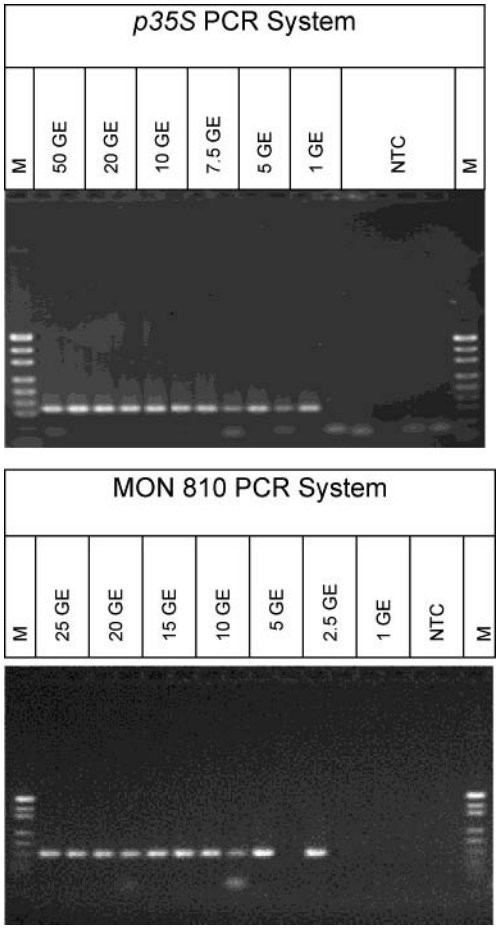

Figure 2. Sensitivity tests of the 123 bp *p35S* and the 149 bp MON 810 PCR systems. Serial dilutions of MON 810 DNA were tested. To compare the sensitivity results with other PCR systems, the amount of DNA is depicted in genome equivalents (GE) per reaction, which corresponds to haploid genomic copies of MON 810 DNA. NTC: no template control. M (DNA molecular weight marker): 501/498, 409, 325, 242, 190, 147, 110, and 67 bp.

Table 2. Number of Samples Analyzed by Various PCR Assays

	total samples (no.)	*rbcL* (173 bp) (no.)	*rbcL* (500 bp) (no.)	*p35S* (123 bp) (no.)	MON 810 (149 bp) (no.)
pork	118	118	30	118	1
beef	20	20	0	20	0
chicken	20	20	0	20	1
milk	14	14	14	14	0

due to PCR inhibition, each sample PCR was run in parallel with a PCR spiked with positive DNA. The number and type of animal tissue samples initially analyzed by each of the four PCR methods used in this study are shown in **Table 2**.

If a pair of duplicates yielded indeterminate (+/−) results, repeat PCRs were performed on the same duplicate DNA extracts. If the repeat PCR results were again indeterminate, repeat DNA extractions were prepared from two other subsamples, and the resulting duplicate DNA preparations were analyzed by PCR. If this repeated DNA extraction/PCR analysis still yielded unmatched duplicate results (+/−), the result of the analysis of that particular tissue sample was recorded as indeterminate. It is worth noting that repeated indeterminate results are foreseeable when the level of the target DNA in a sample is slightly below the LOD of the assay (*18*−*20*).

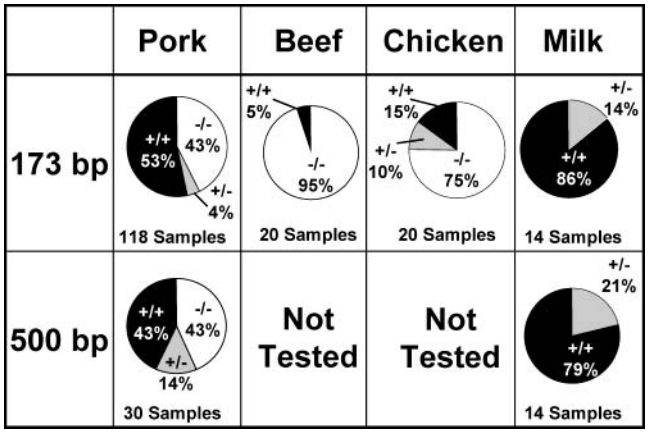

Figure 3. Maize *rbcL* PCR assay results: (black) positive (+/+), (white) negative (–/–), (gray) indeterminate (+/–).

PCR Analysis of Animal Samples for *rbcL* DNA Fragments. Using the 173 bp *rbcL* PCR method, fragments of maize-specific chloroplast DNA were detectable in at least some samples of all tested animal tissue types, although there was a wide range for the percentage of samples that were positive (**Figure 3**). For the beef samples, one out of 20 samples was positive (5%), and for the chicken samples, three out of 20 samples were positive (15%). By comparison, 53% of the pork samples (63 of 118) and 86% of the milk samples (12 of 14) were positive for the maize-specific 173 bp *rbcL* PCR amplification product. The identity of 10 of the 173 bp *rbcL* PCR products (derived from across the various sample types) was verified by sequence analysis, showing them to be identical with the known maize *rbcL* reference sequence (data not shown).

To better understand the size of the DNA fragments being detected in the pork and milk samples by the chloroplast *rbcL* PCR assay, a subset of the positive pork samples (30) and all 14 of the milk samples were analyzed with the 500 bp *rbcL* PCR method (**Figure 3**). The 500 bp *rbcL* amplicon was positively detected in 79% of the milk samples (11 of 14) and in 43% of the pork samples (13 of 30). Thus, it appears that the 173 bp *rbcL* PCR method detected DNA fragments in a higher percentage of the pork samples than the 500 bp *rbcL* PCR. This result could be due to the different sensitivity levels of these two *rbcL* PCR assays, but it is also consistent with an expectation that DNA is undergoing degradation during digestion and smaller fragments are more prevalent than larger ones.

To determine the repeatability of these *rbcL* results, new subsamples of 31 previously tested positive pork samples were subjected to DNA extraction and analysis with both *rbcL* PCR assays. As shown in **Figure 4**, 58% of the samples that had previously tested positive in the 173 bp *rbcL* PCR were again positive in this assay when new aliquots were subjected to DNA extraction and analysis. This repeat analysis also showed 23% of the samples gave indeterminate results and 19% were negative. Because different aliquots of the same pork samples were used in the two PCR analyses, it is possible that unequal distribution of the plant DNA fragments in the pork muscle tissues explains the differing results. These differing results could also reflect the difficulty to repeatedly detect rare plant DNA fragments in animal tissues. It is noteworthy, however, that the 500 bp *rbcL* amplification product was detectable only in samples in which the 173 bp *rbcL* PCR method had also shown positive results. In addition, all of the samples that were negative with the 173 bp method were also negative with the 500 bp *rbcL* PCR method.

The detection of fragments of the maize *rbcL* gene in some animal tissue types by PCR is generally consistent with

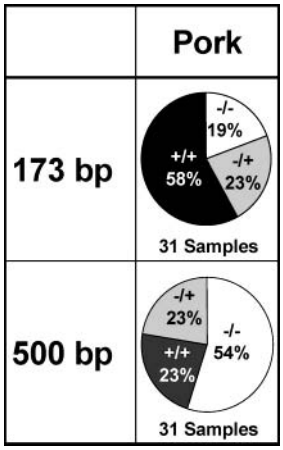

Figure 4. Repeat maize *rbcL* PCR assays results for pork muscle samples: (black) positive (+/+), (white) negative (–/–), (gray) indeterminate (+/–).

previously published results, although there are some notable differences in the number of detects for some tissue types. In two previous reports (*9*, *10*), a 199 bp fragment of a highly abundant chloroplast gene, $tRNA_{leu}$, was detectable in different chicken tissue samples and not significantly detectable in muscle tissue samples from beef steers, which is similar to the findings of the present study. It has also been reported that a 140 bp fragment of the *rbcL* gene was detected by PCR in 33%, 54%, and 23% of samples from the musculus gluteus maximus, musculus longissimus dorsi, and musculus trapezius, respectively, of swine fed Bt or conventional maize (*11*). In contrast to the present results with milk samples, in which nearly all of the samples were positive for both the 173 and 500 bp DNA targets, one prior report had only marginally detected DNA fragments of a chloroplast gene in milk (*9*). One simple explanation for the dissimilar results could be differences in analytical methodology (e.g., DNA extraction methods, PCR primers, and PCR conditions).

Second, for dairy milk samples, the extreme difficulty in collecting samples free of plant-derived dust, abundant in the dairy environment and on the cows, is acknowledged, especially given the extreme sensitivity of the present PCR methods for high copy number genes such as *rbcL*. Because of the potential for laboratory-derived contamination, in this study both the PCR analyses and also the extraction procedures were controlled by inclusion of extraction-negative and PCR-negative controls. The importance of stringently controlled experimental procedures during analyses of high copy number genes was described in a recent report (*21*). The authors showed that, working under standard laboratory conditions, PCR methods similar to those used in this study detected a *rbcL* gene fragment in 48% of the tested bovine tissue samples. By comparison, using stringently controlled laboratory conditions, the same PCR methods detected the plant-derived DNA fragment in only 3% of the same samples.

In this study, the most notable differences in detectability of the maize *rbcL* gene did not occur between ruminants and monogastric animals, as anticipated, but within these groups. While *rbcL* fragments were detected in muscle tissue from two monogastric species (chickens and swine), the frequency of detection in pork samples was significantly greater than that in chicken samples. This difference may be due to the differences in the digestive physiology of the two species or due to differences in muscle samples, time of feeding, or other factors.

PCR Analysis of Animal Samples for Transgenic DNA Fragments. A key question for this study was to determine if sensitive PCR methods could detect transgenic plant DNA fragments in tissue samples from animals fed these GM products. Therefore, all animal samples were analyzed by PCR for a 123 bp DNA fragment of the CaMV 35S promoter (**Table 2**). In case of a positive or indeterminate result, the samples also were tested with a PCR method using a completely different set of primers specific for MON 810 (the "MON 810 PCR").

All of the 14 milk and 20 beef samples tested negative for the 123 bp fragment of *p35S*. One chicken sample (C18) out of the 20 tested samples repeatedly produced an indeterminate (+/−) PCR result for the 123 bp DNA fragment of *p35S*. Because the C18 sample was from a chicken fed conventional, nonGM maize, this indeterminate result should not be interpreted to suggest the presence of DNA fragments from ingesting MON 810 maize. This is supported by the fact that further analysis of sample C18 by the MON 810 PCR method testing for a 149 bp amplicon was also negative. One pork sample (P13) of 118 samples produced a positive result in the *p35S* PCR assay. The P13 sample was from one of 59 animals that had been fed MON 810 maize. For additional confirmation of the positive *p35S* PCR result of pork sample P13, the analysis was repeated with new tissue subsamples, which again showed positive results. Further analysis of pork sample P13 with the MON 810 PCR method yielded indeterminate (+/−) results, suggesting that the number of target copies in the sample were below the detection limit of the method.

Because these PCR data show a binomial distribution (e.g., either positive or negative for the test DNA) with a modest number of samples, the Pearson Chi-Square Exact statistical test (*17*) was used to further understand the *p35S* PCR assay for the control (conventional maize) and the test (MON 810 maize). This statistical analysis showed that the probability (P_R) that the *p35S* PCR results for the pork samples from animals fed conventional maize and samples from animals fed MON 810 maize were similar was 1.000. This indicates that the positive detection with sample P13 was not statistically significant, because $p \leq 0.05$ for 59 total samples indicates that 2−3 positive results can be observed by random chance alone.

The identity of the *p35S* PCR products of chicken sample C18 and pork sample P13 was verified by sequence analysis, showing them to be identical with the CaMV 35S promoter reference sequence (GenBank Accession No. V00141) except for one mismatch in the sequence of C18 (data not shown).

Summary. Transgenic DNA fragments were not detected in any of the tested animal tissue samples, including dairy milk and samples of muscle (meat) from chickens and beef steers by the *p35S* PCR. The large number of muscle samples collected from 118 swine for PCR analysis was of sufficient size to conclude that no statistically significant detections of transgenic DNA occurred with this set of tissue samples ($p < 0.05$). By comparison, the 173 bp *rbcL* assay detected the target plant chloroplast DNA fragment in 5%, 15%, and 53% of the muscle samples from beef steers, broiler chickens, and swine, respectively, and in 86% of the dairy milk samples. The 500 bp *rbcL* assay detected DNA fragments in 43% of the swine muscle samples and 79% of the milk samples.

Taken together, these analyses suggest that the gene copy number might be a significant factor influencing the detectability of plant DNA sequences in animal tissues. A single plant cell contains up to 500−50000 copies (roots versus leaves, respectively) of the chloroplast genome (*12*) as compared to the single copy per haploid genome of the transgenic DNA in the nuclear genome of MON 810 maize. The considerably greater abundance of copies of the target chloroplast DNA relative to the target genomic DNA in plant cells essentially provides the *rbcL* PCR assays with a proportional increase in probability of larger

or intact fragments of the more abundant DNA target surviving the digestive tract of animals. It should be noted, however, that, on a mass basis, total nuclear genomic DNA is much more abundant than chloroplast DNA, suggesting that methods to detect high copy number, nuclear-encoded sequences may also yield positive detects.

A recent report showed that a PCR assay failed to detect a 211 bp fragment of the Bt gene in 324 samples collected from nine tissues that had been collected over a 72 h period after feeding swine Bt maize, but a high percentage of these samples were positive for a high copy number plant chloroplast gene (*11*). Together, the present data plus the data from these other reported studies show that the presence of DNA fragments of high copy number plant sequences in some animal tissues appears to be a normal metabolic occurrence that is variable between different animal species and between tissues types within animal species.

The variability of the detection of the high copy number targets and the absence of significant detection of single copy DNA targets is consistent with the expectation that DNA is undergoing rapid degradation in the digestive tract of animals. Follow-up studies could be designed to further elucidate the mechanism that results in the presence of detectable levels of high copy number plant DNA fragments in tissues from some animals.

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