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Detection of Transgenic and Endogenous Plant DNA Fragments in the Blood, Tissues, and Digesta of Broilers

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The aim was to determine the fate of transgenic and endogenous plant DNA fragments in the blood, tissues, and digesta of broilers. Male broiler chicks ($n = 24$) were allocated at 1 day old to each of four treatment diets designated T1−T4. T1 and T2 contained the near isogenic nongenetically modified (GM) maize grain, whereas T3 and T4 contained GM maize grain [*cry1a(b)* gene]; T1 and T3 also contained the near isogenic non-GM soybean meal, whereas T2 and T4 contained GM soybean meal (*cp4epsps* gene). Four days prior to slaughter at 39−42 days old, 50% of the broilers on T2−T4 had the source(s) of GM ingredients replaced by their non-GM counterparts. Detection of specific DNA sequences in feed, tissue, and digesta samples was completed by polymerase chain reaction analysis. Seven primer pairs were used to amplify fragments (~200 bp) from single copy genes (maize high mobility protein, soya lectin, and transgenes in the GM feeds) and multicopy genes (poultry mitochondrial cytochrome b, maize, and soya rubisco). There was no effect of treatment on the measured growth performance parameters. Except for a single detection of lectin (nontransgenic single copy gene; unsubstantiated) in the extracted DNA from one bursa tissue sample, there was no positive detection of any endogenous or transgenic single copy genes in either blood or tissue DNA samples. However, the multicopy rubisco gene was detected in a proportion of samples from all tissue types (23% of total across all tissues studied) and in low numbers in blood. Feed-derived DNA was found to survive complete degradation up to the large intestine. Transgenic DNA was detected in gizzard digesta but not in intestinal digesta 96 h after the last feeding of treatment diets containing a source of GM maize and/or soybean meal.

KEYWORDS: Genetically modified feeds; transgenic DNA; polymerase chain reaction; blood; tissues; digesta; broilers

INTRODUCTION

The global area sown to genetically modified (GM) crops has increased rapidly from 1.7 million ha in 1996 to 81 million ha in 2004 (*1*). Presently, about 98% of the global GM crop production is contained in the United States, Argentina, Canada, Brazil, China, and Paraguay, with the United States being the predominant producer (47.6 million ha in 2004) (*1*). In these countries, the principal GM crops, modified mainly for herbicide tolerance and/or insect resistance, are soybeans, maize, cotton, and canola, accounting for 60, 23, 12, and 5% of the global GM area in 2003, respectively (*1*).

Numerous studies (e.g., ref *2*) have confirmed the compositional and nutritional equivalence (see ref *3* for description) of GM crops when compared with their isogenic non-GM or conventional counterparts. However, the inclusion of GM crops in livestock rations, as the whole crop (e.g., maize silage) or as a specific crop component (e.g., maize grain) or coproduct (e.g., oilseed meals), has raised a number of safety concerns including the potential for transgenic DNA (tDNA) and/or protein to transfer to animal-derived products intended for human consumption. For such a phenomenon to occur, however, requires initially that animal feeds should contain undegraded or "intact" DNA and that a proportion of feed DNA must survive complete digestion in the gastointestinal (GI) tract of various animal species.

Until only recently it was generally assumed that most feed DNA was destroyed or rendered "inert" by processing. However, it has been reported (*4*, *5*) that processing has a variable effect on the extent of feed DNA degradation. Smith et al. concluded that the extent of DNA degradation was generally related to the method of processing, with processes involving both heat and chemical treatment having the greatest effect. Under experimental conditions (*4*), it was observed that high temperatures (at least 95 °C) together with pressurized steam were required to fully degrade genomic DNA in wheat and maize.

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Table 1. Ingredient Composition (g/1000 g Fresh Weight) of the Treatment Diets

feed ingredient	starter (0–2 weeks)	grower (2–4 weeks)	finisher (4–6 weeks)
maize grain[a]	600.0	600.0	600.0
poultry fat blend	35.0	30.4	51.2
vegetable oil	7.0	16.6	
soybean meal[b]	250.0	243.8	238.8
fishmeal	80.0	80.0	80.0
mineral/vitamin supplement[c]	5.0	5.0	5.0
synthetic methionine	3.0	3.1	4.4
synthetic lysine	5.6	8.7	
synthetic threonine	0.364		4.6
limestone	10.2	5.9	
dicalcium phosphate 18	1.2	4.8	14.6
sodium chloride	0.454	0.1	
sodium bicarbonate	2.2	1.6	1.4

[a] Non-GM maize (Dekalb hybrid DK626) for T1 and T2 and GM maize (Dekalb hybrid DK626Bty, MON 810) for T3 and T4 (the maize grain was milled though a 3 mm screen using a Christy Norris hammer mill and stored at 3 °C prior to use). [b] Non-GM soybean meal (Bronson soybeans) for T1 and T3 and GM soybean meal (Excel 8382 soybeans) for T2 and T4. [c] Composition per kg: 310 g of Ca, 857 g of ash, 20 mg of Se, 1000 mg of Cu, 1200000 IU of Vit A, 400000 IU of Vit D, and 5000 IU of Vit E.

These findings suggest, therefore, that the ingestion of plant DNA by animals is a natural consequence of eating.

Recently, attention has focused on determining the fate of ingested tDNA including transfer to animal products such as milk (e.g., ref *6*) and meat [e.g., (broilers) *7*−*10*; (laying hens) *7*; and (pigs) *11*]. To date, there have been no reports of the positive detection of specific transgene sequences in the tissues or organs of animals fed GM-based diets (see ref *3* for review). However, small fragments [∼199 base pair (bp)] of plant chloroplast DNA have been detected in some animal tissues (e.g., *6, 7, 11*−*13*).

The aim of the present study was to determine the fate of feed DNA (including tDNA) in broilers by analysis of digesta collected from the GI tract and analysis of tissues removed from the carcasses of birds consuming diets containing GM feed ingredients or their near isogenic non-GM counterparts. The study also aimed to determine the effect of withdrawing the source of GM feed ingredients 4 days prior to slaughter on tDNA and endogenous DNA detection.

MATERIALS AND METHODS

Experimental Treatment Diets. Four experimental treatment diets, designated T1−T4, based on GM or non-GM maize grain and soybean meal (principal energy and protein sources, respectively) were prepared: T1 (control), non-GM maize and non-GM soybean meal; T2, non-GM maize and GM soybean meal; T3, GM maize and non-GM soybean meal; and T4, GM maize and GM soybean meal. The GM maize grain contained the *cry1a(b)* gene for Monsanto YieldGard Bt Maize event MON 810 and the GM soybean meal *cp4epsps* gene for Monsanto Roundup Ready Soybean event GTS-40-3-2. Dekalb maize hybrid DK626 and Bronson soybeans were the near isogenic (non-GM) counterparts. The treatment diets were reformulated during the feeding study to meet the nutritional requirements of the broilers from 0 to 2, 2 to 4, and 4 to 6 weeks of age, designated starter, grower, and finisher, respectively (see **Table 1**). The treatment diets were prepared just prior to the start of each 2 week period and stored at ∼3 °C.

To prevent any cross-contamination of the treatment diets, stringent quality control procedures were employed throughout their preparation including cleaning all surfaces, instruments, and equipment with ethanol and/or Klorsept solution; milling all of the non-GM maize grain prior to the GM maize; use of dedicated equipment for handling and weighing all GM and non-GM ingredients; and wearing different protective clothing for all handling of GM and non-GM ingredients and treatment diets.

Broiler Feeding Study. One hundred twenty as hatched male broiler chicks (Ross 308s; Highline Turkeys Ltd., Shropshire, United Kingdom) were randomly allocated at 1 day old to one of 20 poultry pens (six chicks per pen; five pens per treatment diet) in order to achieve approximately the same initial group live weight in each pen. Each pen had a floor area of 1 m^2 and a volume of 1 m^3. The litter was comprised of ∼4 cm depth of clean wood shavings. One pen in each treatment group was designated as a source of replacements of any bird(s) removed (e.g., through ill health) from the remaining four pens during the course of the feeding study.

Each pen contained at least one fount water drinker, and water was freely available throughout. The air temperature in the pens was gradually reduced from ∼29 °C on day 1 until 21 °C was reached by day 21. The light intensity was gradually reduced from the maximum attainable on day 1 (∼40 lux) to approximately 10 lux by 10 days of age. The lighting schedule throughout the feeding study was 23, 16, and 23 h of light/24 h from 0 to 4, 5 to 21, and 22 to 42 days of age, respectively. All birds were observed at least twice daily for signs of disease, illness, or injury. Any broiler removed from the study was weighed, and a record was kept of the reason for its removal.

The dietary treatments were fed in the form of a crumb from 0 to 6 weeks. After the initial period of introduction, the daily amount of feed offered was ∼110% of the previous day's intake in order to allow ad libitum intake. Six birds from each treatment group were slaughtered at 39, 40, 41, and 42 days old (i.e., total 24 birds per treatment). For treatment diets T2−T4, 50% of the birds stayed on their respective GM diets throughout the feeding study, while for the remaining birds the dietary source(s) of the GM ingredient(s) was removed and replaced with the non-GM control diet (T1) 4 days prior to slaughter at 39−42 days old (designated T2/T1−T4/T1).

Slaughter and Sampling of Animal Tissues and Digesta. Immediately following slaughter (dislocation of the neck), the area surrounding the wing vein was cleaned with ethanol and then a blood sample (∼2 mL) was taken using a separate sterile needle and syringe for each bird. The blood sample was then transferred to a sterile 15 mL polypropylene tube containing ∼0.2 mL of sodium citrate solution (4%, w/v), and the tubes kept on ice until the samples were processed.

After weighing, the bird's throat was cut to allow bleeding and was then hung upside down at ∼3 °C for 15 min. Each bird was then carefully dissected to obtain the appropriate tissue and digesta samples (see **Tables 6** and **7**). All samples were placed in separate labeled tubes and immediately frozen at −80 °C.

Sample Preparation for DNA Analysis. *Feed Samples.* A representative sample each of the non-GM and GM maize and soybean meal and the four treatment diets was prepared by finely grinding in liquid nitrogen using clean, sterile mortars and pestles (prepared using Klorsept solution). The ground material was then placed in labeled plastic tubes and stored at −80 °C.

Blood. Each whole blood sample was carefully transferred onto the upper surface of 1 mL of Histopaque solution (Sigma) in a 15 mL conical tube. The tubes were centrifuged at 400*g* for 30 min. The upper layer, representing serum, and the layer of mononuclear cells (white blood cells, WBC) that formed at the interface with the Histopaque solution were carefully removed to separate sterile 1.5 mL microcentrifuge tubes and then stored frozen (−80 °C).

Tissue Samples. All tissues and organs were defrosted, and then, 2 g of breast tissue, liver, and gizzard was taken by cutting out an internal portion of the tissue with a clean scalpel blade. The entire sample of heart, spleen, kidney, and bursa was processed. For the heart, this involved removing any traces of fat adhering to the outer surface of the organ prior to dissecting it to expose the internal tissue. Any clotted blood was then carefully removed before immersing the entire heart several times in 30−40 mL of sterile phosphate-buffered saline (PBS) to remove the remaining clotted blood. The entire heart was weighed and then chopped into small pieces using scissors.

Each tissue was mechanically disrupted (for 2.5 min for liver, spleen, and kidney; 5 min for breast tissue; 6 min for bursa; 8 min for gizzard; and 15 min for heart) using an Ultra Turrax T25 tissue homogenizer

Table 2. Primer Pairs Used for the Detection of Single and Multicopy Genes[a]

primer name	sequence (5′-3′)	amplicon size (bp)	specific detection	ref
ADRUB F1 ADRUB R1	ttc caa ggt ccg cct cac cat cat ctt tgg taa aat caa gtc c	167	maize and soya bean rubisco (multicopy) gene	1
ADLECT F1 ADLECT R1	cca caa aca cat gca ggt tat c aga cca aag aag caa cca aga g	240	soya bean lectin (single copy) gene	1
ADHMP F1 ADHMP R1	ttg tgt gga ttg tag gac aag g gct ctg aac aaa caa cac atg g	209	maize HMP (single copy) gene	1
Cyt B F1 Cyt B R1	cat ccc caa caa act tgg ag tac tgg ttg gct tcc gat tc	180	poultry cytochrome b (multicopy) gene	2
MON 810 F1 MON 810 R1	ctc cct agt gtt gac cag tgt tac ctg cgt gag gga gag gga gat gtc	203	transgenic *cry1a(b)* (single copy) gene	3
35S F3 CTP R1	cga tgt gat atc tcc act gac g tgt atc cct tga ccc atg ttg t	171	transgenic *cp4epsps* (single copy) gene	4
35S F4 35S R1	gct cct aca aat gcc atc a gat agt ggg att gtg cgt ca	195	transgenic 35S promoter gene (single copy) from CaMV	5

[a] Refs: 1, ref *6*; 2, developed by the authors; 3, Monsanto Co.; 4, ref *15*; and 5, Promega.

plus a tissue dispersal tool (8 mm diameter), in the presence of sodium dodecyl sulfate (SDS)−cell lysis buffer [CLB; 10 mM Tris, pH 8.0, 1% w/v SDS, and 50 mM ethylenediaminetetraacetic acid (EDTA)]. A ratio of CLB:tissue (v/w) of 4:1 was used throughout. All homogenized material was either kept on ice for up to 4 h or immediately stored frozen at −80 °C.

Digesta Samples. A representative subsample of the undigested contents of the gizzard, duodenum, remaining small intestine, and large intestine was prepared by finely grinding in liquid nitrogen using clean, sterile mortars and pestles. The ground material was stored frozen (−80 °C) in fresh, labeled plastic tubes.

DNA Extraction Methodologies. DNA was extracted from each tissue homogentate (600 μL in duplicate) using the SDS−Proteinase K extraction method based on that described previously (*9*). The isolated DNA was resuspended in molecular biology grade water (200 μL for breast, heart, and gizzard tissue; 800 μL for liver, kidney, spleen, and bursa) by incubating for 1 h at 65 °C. For the feed and gizzard digesta samples (25 mg/extraction of soybean meal; 100 mg/extraction of ground maize grain, treatment diet samples and gizzard digesta), DNA was extracted using the cetyl trimethylammonium bromide (CTAB) extraction method (*14*). The extracted DNA was resuspended in 100 μL of molecular biology grade water. The QIAamp DNA Stool Mini Kit was used for the purification of DNA from duodenal, remaining small intestine, and large intestine digesta contents and the QIAamp DNA Blood Mini Kit from WBCs and serum. For both commercial kits, the isolated DNA was eluted from the QIAamp columns in 200 μL of molecular biology grade water.

The extracted DNAs, except that extracted from serum, were quantitated by absorption at 260 nm, and then, all DNAs were stored frozen at −80°C until analyzed by polymerase chain reaction (PCR).

Design of Oligonucleotide Primers. Seven primer pairs (**Table 2**) were used (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3) to amplify specific DNA sequences of ∼200 bp. All DNA oligonucleotides, synthesized by Sigma-Genosys, were resuspended at 50 μM in molecular biology grade water and stored as aliquots at −20 °C. The ADLECT and ADHMP [forward (F) and reverse (R)] primers were used to amplify a specific fragment from the single copy genes coding soya lectin and maize high mobility protein (HMP), respectively. A sequence alignment of the *Zea mays* and *Glycine max* multicopy chloroplast *rbcL* genes was generated using the Blast sequence alignment facility (http://ncbi.nlm.nih.gov/BLAST/), and the data were used to design the ADRUB F/R primers that would amplify both maize and soya *rbcL* gene fragments.

To detect tDNA in Monsanto YieldGard Bt maize, the MON 810 F/R primer sequences (from Monsanto Co., St. Louis, MO) were used to amplify a 203 bp fragment overlapping the Hsp70 intron [preceding the *cry1a(b)* transgene] and the *cry1a(b)* coding region. To specifically detect the transgene in Monsanto Roundup Ready Soybean, the 35S F3 and CTP R1 primer pair, described by Wurz and Willmund, was used. These primers span the 35S promoter and chloroplast transit peptide of the inserted transgene. In addition to the transgene specific primers, the 35S F4 and R1 primers were used to amplify the cauliflower mosaic virus (CaMV) 35S promoter element that drives the expression of the transgenes in the two GM plants. For all of the tissue DNA samples, the Cyt B F/R primers were used to detect the poultry (*Gallus gallus*) mitochondrial cytochrome b gene. Because this gene is ubiquitous in all poultry tissues, the results of the PCR amplification of the cytochrome b gene sequence were used to assess the presence of PCR inhibitors (e.g., lipid, carbohydrate, divalent cations) and therefore the suitability of the extracted DNA for PCR analysis. DNA was reextracted from any tissue sample(s) where cytochrome b was not detected.

PCR Methodologies. Prior to PCR, the extracted DNA was diluted in molecular biology grade water to give either 100 ng of DNA (from WBCs and all feed and digesta samples) or 500 ng of DNA (from all tissue samples) per 5 μL. In the case of the serum DNA, 5 μL of the undiluted DNA sample was used in each PCR. For every amplicon, a single PCR was carried out on each of the two extracted DNAs from each sample. Prior to the start of the study, PCR acceptance/rejection criteria were established (**Table 3**) and failure to meet any of these criteria invalidated the entire amplicon set on the PCR plate. Using these criteria, each replicate DNA was scored positive (+) or negative (−) for each of the seven amplicons and then from these results each sample was then scored positive (+, +), negative (−, −) or inconclusive (+, −) for all amplicons (only the number of positive detections is reported).

The PCR mixes contained per reaction: 2 mM magnesium chloride, 200 μM deoxynucleotide triphosphate (dNTP) mix (Abgene), 1 μM each PCR primer, 1.25 units of "Red Hot" DNA polymerase (Abgene), and 5 μL of diluted DNA in a final volume of 50 μL of 1×PCR buffer. Reactions were assembled in a UV-irradiated PCR workstation. For each amplicon on every PCR plate, there was a positive control (5 ng of GM maize /soybean meal DNA; 50:50 w/w mix), a negative control (5 μL of water), and an extraction control (duplicate). Additionally, for transgene detection in the tissue samples, "spiked control" reactions were set up by spiking 500, 100, 50, or 5 pg of mix of GM maize or soybean meal DNA into broiler tissues from birds receiving the non-GM treatment diet (T1). PCR plates were "hot started" by placing the PCR plate onto a preheated (94 °C) thermocycler PCR machine (GeneAmp PCR System 9700; Applied Biosystems, Warrington, United Kingdom). The PCR conditions were 94 °C for 2 min, then cycling at 63 °C for 20 s, 72 °C for 20 s, and 94 °C for 20 s, followed by a final soak at 72 °C for 5 min. PCR was carried out for 30 cycles for the detection of cytochrome b and for 40 cycles for all other amplicons.

Agarose Gel Electrophoresis. Isolated genomic feed DNA and the PCR products were separated by electrophoresis using 0.8 and 2% (w/

Table 3. PCR Acceptance and Rejection Criteria

true positive PCR	true negative PCR
1. The presence of correct size PCR product for each amplicon is assessed visually on ethidium bromide-stained agarose gels	1. The absence of correct size PCR product for each amplicon is assessed visually on ethidium bromide-stained agarose gel.
2. Positive (5 ng of extracted target DNA) and negative control (water) reactions show expected results.	2. Positive (5 ng of extracted target DNA) and negative control (water) reactions show expected results.
3. Buffer extractions (controls for contamination during extraction) are negative for all amplicons.	3. Buffer extractions (controls for contamination during extraction) are negative for all amplicons.
4. Poultry cytochrome b sequence is amplified in isolated tissue DNA.	4. Poultry cytochrome b sequence is amplified in isolated tissue DNA.
5. Results are consistent with animal diets [i.e., detection of the transgene(s) in samples from animals fed GM treatments].	5. Results are consistent with animal diets [i.e., no detection of the transgene(s) in samples from animals fed non-GM treatments].

Table 4. Summary of Growth Performance Parameters of the Broilers (n = 24/Treatment) Receiving the Four Experimental Treatments Diets T1–T4[a]

	experimental treatment diet				
parameter	T1	T2	T3	T4	significance
initial mean LW (g)	40.6	40.5	39.7	39.7	ns
final LW (g)	1976	2046	2173	2015	ns
LW gain (g/day)	47.7	49.5	52.7	48.8	ns
feed intake (g)	4067	4097	4210	3880	ns
FCR[b]	2.13	2.09	2.00	1.98	ns

[a] Six birds from each treatment group were slaughtered at 39, 40, 41, and 42 days old (total of 24 birds per treatment group). T1, non-GM maize and non-GM soybean meal; T2, non-GM maize and GM soybean meal; T3, GM maize and non-GM soybean meal; T4, GM maize and GM soybean meal; LW, live weight; and ns, not significant ($P > 0.05$). [b] Feed conversion ratio = feed intake (g)/live weight gain (g).

v) agarose gels, respectively, containing 1 μg/mL ethidium bromide. Feed DNA (1−2 μL) was analyzed on 0.8% agarose gels. For PCR products, 20 μL of Bromophenol blue loading dye was added to each reaction and then 20 μL of diluted PCR product was run on 2% agarose gels incorporating 100 bp markers (Abgene). Gels were run at 100 V for 1.5−2 h, and the DNA was visualized under UV light.

DNA Sequencing. A number of the amplified PCR products were sequenced in order to prove their authenticity following gel purification of the appropriate PCR product using a Qiagen MinElute gel extraction kit (Qiagen Genomic Services, Germany). The text sequence was pasted into the Blastn sequence alignment facility to match the sequence data with the information held in the databases. The sequencing information was used to confirm or reject positive PCR results.

Statistical Analysis. Growth performance parameters (e.g., live weight gain) were analyzed by analysis of variance of a randomized block design using MINITAB Statistical Software (v. 13.31).

RESULTS

Broiler Feeding Study. The mean group live weight of the broiler chicks (n = 6) allocated to the pens (n = 4/treatment group) on day 1 was not significantly different ($P > 0.05$) between the four experimental treatment diets (means of 40.6, 40.5, 39.7, and 39.7 g for T1, T2, T3, and T4, respectively). The results given in **Table 4** show that there was no significant ($P > 0.05$) effect of treatment on any of the growth performance parameters measured. However, mean final live weights (g) and live weight gains (g/day) across the treatments were significantly different ($P = 0.021$) on days 40−43 (e.g., live weight gain, 1879, 2002, 2042, and 2126 g/day for days 40−43, respectively). There was also a significant effect ($P = 0.028$) of broiler age at slaughter (days 40−43) on the calculated feed conversion ratio.

Feed DNA Extraction and Fragmentation. The amplification of single and multicopy gene fragments in the GM soybean meal and maize grain samples is summarized in **Table 5**. PCR

Table 5. Detection of Single and Multicopy Genes in the GM Soybean Meal and Maize Grain Studied[a]

specific detection	GM soybean meal	GM maize grain
rubisco	+	+
lectin	+	−
HMP	−	+
transgenic *cry1a(b)*	−	+
transgenic *cp4epsps*	+	−
transgenic 35S promoter	+	+

[a] +, Positive detection; −, negative detection.

analysis also confirmed the presence of the *cp4epsps* and *cry1a-(b)* constructs in treatment diets T2 and T4 and T3 and T4, respectively, and the CaMV 35S promoter gene in the three treatments diets containing GM ingredients (T2−T4).

The non-GM soybean meal and maize grain samples, intended to be near isogenic, were shown, however, to contain trace amounts of their GM counterparts (data not shown). A low level detection of *cp4epsps* was also reported for treatment diet T3 containing no GM soybean meal. To confirm these findings, samples of the non-GM soybean meal (n = 1), maize grain (n = 2), and treatment diet T1 (non-GM; n = 1) were analyzed at an independent laboratory [Reading Scientific Services Laboratory (RSSL), Reading, United Kingdom]. Using a semiquantitative PCR assay, no GM maize (as measured against a Bt 176 maize standard) was found in the non-GM maize samples; however, a low level (<0.1%) of GM maize DNA was detected in one of the two maize samples when a nonquantitative assay for the detection of *cry1a(b)* was used. Semiquantitative PCR analysis also confirmed that the non-GM soybean meal sample contained 0.1 to <1% GM soya and that treatment diet T1 contained a low level of *cp4epsps* (<0.1%) and *cry1a(b)* (0.1 to <1%).

The agarose gel electrophoresis (0.8% w/v) results for the genomic DNA extracted from the GM and non-GM maize grain and soybean meal samples are illustrated in **Figure 1**. DNA extracted from the GM and non-GM maize grain (3 mm milled) was present in fragments greater than 23 kilobase (kb) while that from the GM and non-GM soybean meal samples was visible as a smear of degraded DNA fragments from 2 kb to <500 bp.

Detection of Feed Derived DNA by PCR. *WBCs and Serum.* There was no positive detection of any of the endogenous or transgenic single copy genes in either WBC or serum samples. Fragments of the multicopy *rbcL* gene were amplified in a low number of the WBC and serum samples (one in 66 and seven in 89 samples, respectively).

Tissue Samples. The number of positive PCR results for each amplicon in each tissue type is summarized in **Table 6**. No endogenous or transgenic single copy plant genes was detected

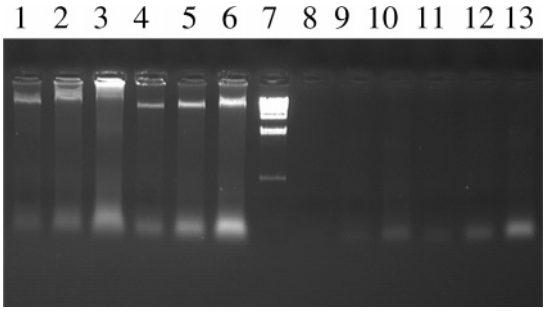

Figure 1. Fragmentation of DNA from maize grain and soybean meal. Lane 7, λ Hind III digest; lanes 1–3, non-GM maize grain DNA; lanes 4–6, GM maize grain DNA; lanes 8–10, non-GM soybean meal DNA; lanes 11–13, GM soybean meal DNA. DNA is loaded at 1, 2, and 4 μg for each sample set.

Table 6. Positive Detections of the Multicopy Rubisco Gene in the Range of Broiler Tissues Examined[a]

	treatment diet							detections	
tissue type	T1	T2	T2/T1	T3	T3/T1	T4	T4/T1	no. +	% +
heart	7	1	0	2	5	2	0	17	18
liver	2	1	2	2	0	2	0	9	9
kidney	13	2	2	2	1	6	1	27	28
bursa	5	9	2	2	8	5	1	32	33
spleen	5	2	0	5	5	1	1	19	20
breast	7	3	1	3	0	1	1	16	17
gizzard	8	9	9	9	1	0	1	37	39
overall total	47	27	16	25	20	17	5	157	23

[a] T1, non-GM maize and non-GM soybean meal; T2, non-GM maize and GM soybean meal; T3, GM maize and non-GM soybean meal; T4, GM maize and GM soybean meal; T2-T4/T1, source(s) of GM material in the diets replaced by non-GM source(s) 96 h prior to slaughter; no. + detections, number of positive detections; % + detections, % of positive detections (number of positive detections expressed relative to a total of 96 for each tissue type).

in any of the tissue DNA samples examined except for one observation (unsubstantiated by sequencing). The single positive result (i.e., one positive PCR result in a total of 3360 PCRs undertaken for the single copy genes) was recorded for lectin in the bursa of a broiler on treatment diet T3. However, the multicopy rubisco gene was detected in a proportion of samples from each tissue type. Irrespective of treatment, the proportion of positive rubisco detections in the range of tissues was 23%. An example of the detection of rubisco in breast, bursa, and spleen tissue samples is illustrated in **Figure 2**.

Digesta Samples. **Table 7** gives the number of positive detections for all amplicons in each digesta type, and their detection in large intestine digesta is illustrated in **Figure 3**. DNA fragments from the single copy lectin and HMP genes were detected in a proportion of each of the broiler digesta samples analyzed but not in duodenal digesta. The transgenes, *cry1a(b)* and *cp4epsps*, were similarly detected in the digesta samples and were consistent with the inclusion of GM maize and soybean meal in the treatment diets.

The number of positive detections of tDNA in the digesta samples from the remaining small intestine and large intestine was low, and no tDNA fragments were present in the same samples from birds where the source of GM material had been removed prior to slaughter. However, tDNA was detectable in gizzard digesta 96 h after the last feeding of diets containing GM ingredients but the number of positive detections was generally lower than in birds consuming GM-based diets until slaughtered.

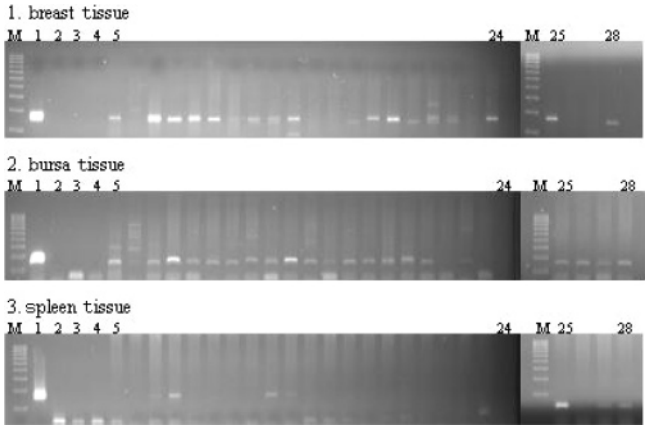

Figure 2. Detection of rubisco in breast, bursa, and spleen tissue samples. M, 100 bp marker; lane 1, positive control; lane 2, negative control; lanes 3–4, extraction controls; lanes 5–28, PCRs on tissue DNA samples (reactions run in duplicate).

Table 7. Positive Detections of Specific Single and Multicopy Genes in the Range of Broiler Digesta Samples Examined[a]

		treatment diet							detections	
digesta type	specific gene	T1	T2	T2/T1	T3	T3/T1	T4	T4/T1	no. +	% +
gizzard	rubisco	12	0	8	8	7	7	5	47	49
	lectin	5	10	7	3	4	4	3	36	8
	HMP	6	11	7	4	4	4	2	38	40
	Cry1a(b)	0	0	0	7	0	4	0	11	15
	cp4epsps	0	11	7	0	2	4	1	25	35
	35S	0	8	4	2	0	4	0	18	25
duodenal	rubisco	13	9	12	10	7	7	8	66	69
	lectin	0	0	0	0	0	0	0	0	0
	HMP	0	0	0	0	0	0	0	0	0
	Cry1a(b)	0	0	0	0	0	0	0	0	0
	cp4epsps	0	0	0	0	0	0	0	0	0
	35S	0	0	0	0	0	0	0	0	0
remaining small intestine	rubisco	22	12	12	12	12	12	12	94	98
	lectin	1	3	1	0	2	0	0	7	7
	HMP	7	5	7	3	11	3	5	41	43
	Cry1a(b)	0	0	0	2	0	0	0	2	3
	cp4epsps	0	2	0	0	0	0	0	2	3
	35S	0	1	0	2	0	1	0	4	6
large intestine	rubisco	22	11	11	10	12	10	8	84	88
	lectin	0	1	0	0	0	2	1	4	4
	HMP	11	5	6	3	7	5	7	44	46
	Cry1a(b)	0	0	0	1	0	0	0	1	1
	cp4epsps	0	1	0	0	0	3	0	4	6
	35S	0	2	0	1	0	6	0	9	13

[a] T1, non-GM maize and non-GM soybean meal; T2, non-GM maize and GM soybean meal; T3, GM maize and non-GM soybean meal; T4, GM maize and GM soybean meal; T2-T4/T1, source(s) of GM material in the diets replaced by non-GM source(s) 96 h prior to slaughter; no. + detections, number of positive detections; % + detections, % of positive detections (number of positive detections expressed relative to a total of 96 for rubisco, and lectin, and 72 for *Cry1a(b), ep4epsp*, and 35S).

Rubisco DNA fragments were detected in each type of digesta DNA studied (see **Table 7**). The proportion of positive detections of rubisco was high in both the remaining small and large intestine digesta samples (98 and 88% of total PCRs, respectively); however, the number of positive detections was lower in the gizzard and duodenal digesta (49 and 69% of total PCRs, respectively).

DISCUSSION

Broiler Feeding Study. In the present study, there was no significant ($P > 0.05$) effect of treatment on the growth

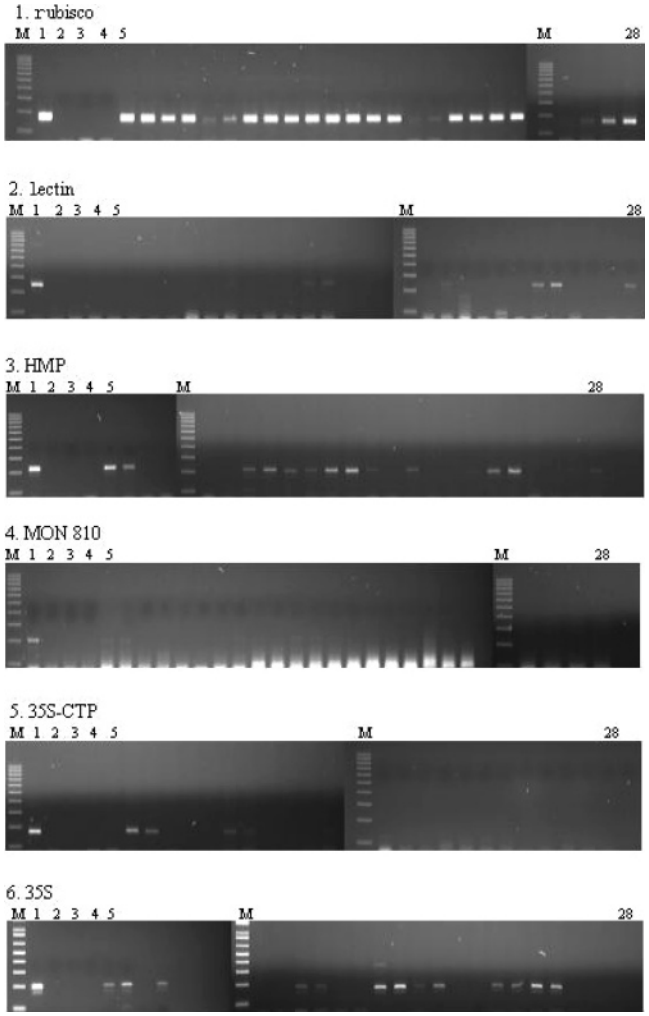

Figure 3. DNA detection in the digesta from the large intestine of broilers. M, 100 bp markers; lane 1, positive control; lane 2, negative control; lanes 3–4, extraction controls; lanes 5–28, respective PCRs on large intestine DNA samples.

performance parameters measured. This finding agrees with the results of previous studies (see review *3* summarizing comparative digestion and feeding studies in poultry, pigs, ruminants, and other food-producing animals) that have confirmed the compositional and nutritional equivalence of GM crops as compared to their isogenic non-GM varieties. Comparative studies (*16*, *17*) with broilers fed Bt GM maize (MON 810) and its conventional counterpart also showed that there were no differences ($P > 0.05$) in the measured growth performance parameters of feed intake, daily gain, or feed conversion efficiency. No significant difference in growth performance parameters between broilers receiving a non-GM diet or a GM maize-based diet (Bt 176) has also been reported (*7*, *10*). In common with a number of authors, Brake et al. stated that "transgenic maize [Syngenta Seeds, Northrup King Bt hybrid N7070] had no deleterious or unintended effects on production traits [e.g., growth, feed conversion ratio, mortality] of broiler chickens' when compared to its isogenic line."

Feed DNA Extraction and Fragmentation. Electrophoresis of the isolated genomic maize DNA (GM and non-GM) showed that the DNA was intact (i.e., >23 kb). According to Smith et al., this is the expected size of plant genomic DNA that has not undergone any processing except for the laboratory DNA extraction procedure. The DNA purified from the GM and non-GM soybean meal samples, visible on the original agarose gel as a smear of DNA fragments from 2 kb to less than 500 bp (**Figure 1**), was shown, however, to have been degraded during the industrial process of oil extraction from whole soya beans. This observation is also in agreement with reports (*4*) on the fragmentation of feed DNA during experimental-scale processing. Following the analysis of a range of unprocessed and processed animal feeds, Smith et al. concluded that the extent of feed DNA fragmentation was related to the degree of processing; DNA fragmentation was greater in feeds processed using both heat and chemical treatments rather than those processed by physicals means alone (e.g., milling).

Knowledge of the extent of feed DNA fragmentation is important since it has implications for the relative level of DNA detection in studies conducted to determine the fate of tDNA in vivo. A highly fragmented DNA will, for example, contain fewer specific amplifiable DNA sequences (i.e., "target" DNA) of the correct size than in nondegraded DNA.

Difficulties associated with sourcing 100% GM-free feeds intended to be isogenic to their GM counterparts are reflected in the fact that a trace amount (<0.1−<1%) of GM material was detected in the non-GM feeds used in this study. Because this observation was also recorded for samples taken prior to opening any of the containers of GM ingredients, then it seems highly likely that any inclusion of GM material in the non-GM feeds occurred prior to shipment of the samples to the United Kingdom. Overall, however, the detection of any GM material in the non-GM feeds/diet was extremely low and therefore does not compromise the findings of the present study. The results obtained for treatment diet T1 appear to be in slight contrast to those obtained for the non-GM soybean meal and maize grain. For soybean meal, the level of GM material was recorded to be 0.1 to <1%, while in treatment diet T1 the results suggest a level of <0.1%. This is likely to reflect the dilution of any GM soybean meal by the remaining dietary components and therefore decreasing the relative concentration of GM material in the diet to below the lowest recorded level (i.e., <0.1%). Conversely, analysis of T1 showed that it contained a slightly higher level (0.1 to <1%) of GM maize than was recorded for the non-GM maize grain. This may reflect the relatively nonhomogeneous nature of the treatment diet or the relatively large particle size of the maize grain (3 mm milled) in relation to the sample taken for analysis. These factors may have allowed the nonhomogeneous distribution of GM material in the sample, thereby increasing the detection of GM maize above the 0.1% threshold. However, overall, the detection of any GM material in the non-GM feeds/diets was extremely low.

Detection of Feed Derived DNA by PCR. *Blood and Tissue Samples.* Within the limits of detection (LOD; 50−100 pg) of the amplicons used to detect GM DNA in blood (WBC and serum) and the range of broiler tissue samples studied, no transgenes were detected. This finding is in agreement with the results of a number of studies investigating the fate of tDNA in vivo (e.g., *6*, *7*, *9*, *13*). In an extensive study (*11*), no tDNA or maize specific DNA could be detected in the blood or tissues (including liver, lymph glands, spleen, kidney, ovary, and three muscle types) of pigs fed a GM maize-based diet ($n = 36$ in GM group). These authors (*11*) stated that "Bt gene fragments are not detectable in tissue samples [from pigs] or, more accurately, are below present limits of detection." This statement emphasizes the need to qualify any negative detection according to the LOD for the specific assay. Other studies have also confirmed that no transgenes could be detected in animal products such as milk (e.g., *6*).

The amplification of fragments of the multicopy rubisco gene in a proportion of each broiler tissue type is consistent with previously reported results (*11*−*13*). Detection of the maize specific chloroplast ivr gene was also recorded (*7*) in organs (liver and spleen), blood, and muscle (weak signal only) in broilers consuming non-GM- and GM-based diets. According to others (*18*), the ability to detect fragments of plant multicopy genes such as rubisco is a function of their copy number; each plant cell can contain 1000s of copies of chloroplast DNA as compared to the two copies of chromosomal genes for a diploid cell. Tony et al. reported that chloroplast gene fragments (199 bp) were detected in the blood, skeletal muscles, liver, spleen, and kidney of broilers but could not be detected in similar samples collected after 24 h of fasting. Reuter and Aulrich calculated the proportion of blood and tissue samples (as %) with amplifiable rubisco DNA fragments (140 bp) to be 17, 54, 17, 13, 27, 37, and 63% for blood, liver, lymph glands, spleen, kidney, muscle (mean of three muscle types), and ovary, respectively. By calculation, the average number of samples (across all tissue types reported) containing amplifiable rubisco fragments was greater in the study of Reuter and Aulrich, involving pigs, than in the present study using broilers (means of 38 and 23% of total observations, respectively). Referring to the detection of chloroplast DNA fragments in pig tissues, these authors (*11*) stated that "the results give evidence that only small particles can pass the epithelial cell layer [of the GI tract and through to the blood stream] of finishing pigs." Further study of multicopy genes (e.g., rubisco) may provide for a greater understanding of the mechanism involved in the possible transfer of plant DNA fragments via the epithelial layer of the GI tract into blood.

Digesta Samples. The detection of the single copy genes in broiler digesta suggests that the complex feed matrix protects the DNA from complete digestion up to the large intestine. This finding is in agreement with others (*10*) who concluded that ingested maize DNA was resistant to complete degradation in the GI tract of broilers and is poorly absorbed. However, Aeschbacher et al. reported that fragments from the Bt 176 specific bla gene were only detected in the crop and not in the gizzard, small intestine, cecum, or excreta. Reuter and Aulrich reported that tDNA was detected in 89, 56, 47, 50, 94, 78, and 83% of digesta samples from the stomach, duodenum, jejunum, ileum, cecum, colon, and rectum, respectively, of pigs up to 12 h after last consuming a diet containing GM maize (70% of total diet). These authors also reported that fragments of tDNA from GM maize were detected in the small intestine (one in six animals) up to 48 h after feeding a GM-based diet. In the present study, fragments of tDNA were detected in gizzard digesta but were not detected in the digesta from the remaining small or large intestine of broilers where the source(s) of GM material had been removed from the treatment diets (i.e., receiving treatment diet T2/T1−T4/T1) 96 h prior to slaughter. However, removing the source(s) of GM material reduced the frequency of tDNA detection in gizzard digesta as compared to those samples collected from broilers fed GM diets until slaughtered. Unlike for all other types of digesta studied, no single copy genes, including transgenes, were detected in duodenal digesta. This observation is probably a reflection of the poor LOD recorded for this digesta type due to the inhibitory matrix effects (e.g., of bile salts) on the PCR assay. The detection of fragments of the *cp4epsps* and 35S genes in the gizzard digesta of two broilers consuming treatment diet T3 and T3/T1 (**Table 7**) (i.e., containing non-GM soyabean meal) is likely to reflect the low level of GM soya detected in the non-GM soyabean meal.

Detection of small fragments of rubisco in broiler digesta is consistent with the findings for other animal species (e.g., *6*, *11*, *12*). In the remaining small and large intestine digesta samples, the proportion of positive detections of rubisco was very high (98 and 89% of total PCRs, respectively), suggesting, as stated for the detection of the single copy genes, that sufficient feed-derived DNA must survive degradation at least until the large intestine in order to be detected by PCR. However, the total number of positive detections was lower in the gizzard and duodenal digesta. While the gizzard digesta were essentially similar to the original dietary material, it is worthy of note that the proportion of rubisco detections was only 49%. This observation may reflect the zero detection of rubisco in the gizzard digesta for broilers on T2 (no explanation can be given for this result) and the use of a different method for the extraction of DNA from gizzard digesta (CTAB) than was used for the remaining digesta types (QIAAmp Stool Mini Kit). Because the CTAB method was used to extract DNA from the feed samples, it suggests that PCR inhibitors may have copurified with the isolated DNA from gizzard digesta. This observation also has consequences for the interpretation of the data relating to the detection of the single copy genes in gizzard digesta. These findings highlight the problems of using different DNA extraction methods across different samples but also the ease with which incorrect conclusions may be derived regarding DNA degradation across the GI tract.

Within the stated LOD of the respective amplicons, it is concluded that no transgenes could be detected in the tissues of broilers consuming GM-based diets. This finding supports the conclusion from the extensive review (*3*) that "no residues of recombinant DNA or novel proteins have been found in any organ or tissue samples obtained from animals fed with GM plants". However, fragments of rubisco DNA could be detected in the tissue types studied. This finding is most likely a function of the abundance of chloroplast DNA in each plant cell but also demonstrates the potential for small DNA fragments to cross the epithelium of the GI tract. The results also show that plant DNA is incompletely degraded in the GI tract of broilers and that DNA fragments of single and multicopy genes can survive complete degradation up to the large intestine. The study also showed that tDNA can be detected in gizzard digesta, but not in intestinal digesta, 96 h after the last feeding of a diet containing a source(s) of GM ingredients. It is concluded that determining the fate of ingested tDNA and/or endogenous DNA is affected by a number of factors including gene copy number, the extent of feed DNA fragmentation, DNA extraction method, sample type and the presence of inhibitors to the PCR assay, and LOD of amplicons.

ABBREVIATIONS USED

bp, base pair; CaMV, cauliflower mosaic virus; CLB, cell lysis buffer; CTAB, cetyl trimethylammonium bromide; dNTP, deoxynucleotide triphosphates; EDTA, ethylenediaminetetraacetic acid; F, forward; GI, gastrointestinal; GM, genetically modified; HMP, high mobility protein; IU, international units; kb, kilobase; LOD, limit of detection; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; R, reverse; SDS, sodium dodecyl sulfate; tDNA, transgenic DNA; UV, ultraviolet; WBC, white blood cells.

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