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Persistence of Plant DNA Sequences in the Blood of Dairy Cows Fed with Genetically Modified (Bt176) and Conventional Corn Silage

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To determine whether plant sequences, including transgenic sequences, are present in animal blood, we tested blood samples from Holstein cows fed with either Bt176 genetically modified corn or conventional corn. We used previously described sensitive real-time PCR assays targeting transgenic sequences (35S promoter and Bt176 specific junction sequence), a monocopy maize-specific sequence (ADH promoter), and two multicopy sequences from plant nucleus (26S rRNA gene) and chloroplast (*psaB* gene). The presence of Cry1A(b) protein in bovine blood samples was also tested using a sandwich ELISA kit. Our study shows the ability of plant nuclear and/or chloroplast DNA fragments to enter bovine blood circulation. However, maize nuclear DNA, both mono- and multicopy sequences, was less detected than chloroplast DNA, probably because the higher number of chloroplast copies and also possibly because nuclear DNA might be less protected by the nuclear membrane. Despite our data confirm the ability of small (ca.150 bp) plant DNA fragments to cross the intestinal barrier, we were unable to demonstrate clearly the presence of transgenic DNA or proteins in bovine blood. No sample tested positive with the two real-time PCR assays targeting transgenic sequences (35S promoter and Bt176 specific junction sequence). Only faint punctual positive results occurred randomly and were probably due to postsample collection or laboratory contamination or can be considered as artifact as they have never been confirmed. Our data highlight the difficulties to detect transgenic sequences in blood of dairy cows fed genetically modified corn (Bt176) silage. Those results show that in order to meet the consumers' demand of animals fed with GM products there is currently no cost-effective analytical procedure to replace documentary traceability.

KEYWORDS: GMO traceability; GMO detection; blood; maize; dairy cow; Bt176; Cry1A(b) protein; real-time PCR; ELISA

INTRODUCTION

The introduction by recombinant DNA technology of new genes into major crops has raised several societal questions in the European Union (EU) and elsewhere. Since few GM crops are grown in the EU, the main consumer questions for keeping their freedom of choice have rapidly shifted toward labeling of GMO derived foodstuff and animals fed with imported GM feed.

Several countries, such as Japan and the EU (directive 2001/18/EEC and regulation 1829/03/EC), enforced mandatory labeling of GM plant derived products. This caused numerous food companies to discard ingredients derived from GM plants or organisms. No country, however, considered the labeling of products from animals fed with GM plants or feed containing GM plants relevant (regulation 1829/03/EC). This caused some

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consumer and environmental organizations to raise the labeling issue for animal products, since the feed concentrates, mainly as soy protein pellets, appeared as the main source of GMOs on the EU market. Along with this request for traceability, there was consumer concern about how DNA sequences from GM plants used as feed could possibly alter animal health and products and in turn eventually pose a threat for human health. Although not scientifically ground, these questions raise scientific issues on the horizontal gene flows between plants (GM or not), microorganisms (GM or not) and animals, including humans.

As far as the request for traceability is concerned, the enforcement of mandatory feed labeling has clarified the issue. In the eyes of the consumer, however (*1*), this traceability is interrupted at the animal stage. With this study, we first attempt to address the pedagogic and economic issues of the fate of feed-ingested DNA from GM plants, in the animal and derived products, and the issue of animal labeling. Within the frame of fraud control on non-GMO claims, we also try to address the feasibility of implementing analytical control methods for detecting analytes (DNA or protein) deriving from GM plants in animals or animal products

The increasing use of GMOs worldwide has resulted in several studies investigating the fate of dietary DNA and proteins from GMOs in animals (*2, 3*). Most dietary DNA is degraded to nucleosides and bases in the gastrointestinal tract (GIT) and then absorbed. It has been demonstrated that small amounts of feed DNA can be transferred into animal tissues and organs (*3*−*6*). Dietary DNA has been found in peripheral leukocytes, spleen, and liver of mice, and also in the fetuses and newborns of mice consuming foreign DNA fragments during pregnancy (*7, 8*). Other studies indicated that DNA from the feed is detectable in various tissues of cattle, chickens, pigs and broilers, showing that ingested DNA has been taken up into the blood (*3, 9*). Those observations imply that fragments of dietary DNA may be found in the bloodstream from the GIT of these animal species. In numerous cases, chloroplast DNA was detected in lymphocytes and duodenal juices, and, as traces, in the milk of cows fed with GM corn. Moreover, short chloroplast sequences were also detected in muscles, spleen, liver and kidney of chickens fed such GM corn (*3*). In 3 additional studies, a DNA fragment from a nuclear plant gene was detected in tissues or milk samples of animals fed GM plants (*3, 10*). Altogether, these results suggest that GM-derived sequences can be detected in animals fed GM. However, in most cases, end-point PCR methods were used without identifying the amplicon which is required by international standards.

In order to contribute to a global reply to these questions, we decided to start a holistic approach by evaluating the putative presence of foreign DNA and protein in blood, a complex animal matrix, carrying nutrients to all animal tissues. Since blood is an intermediate between the digestive tract and animal matrices, the presence or absence of DNA and/or protein in blood would be a good marker of their putative presence in other animal matrices.

Several biotechnology companies (Syngenta, Monsanto, Bayer) associated with the Institut de l'élevage (IE), the Association Générale des Producteurs de Maïs (AGPM), the Centre National Interprofessionnel de l'Economie Laitière (CNIEL) and INRA started some years ago a comparative study of feeding value with sheep and dairy cows (*11*). For several weeks, a herd of 24 cows were fed in 1998 with silage of either conventional or GM (Bt176) corn, in an experimental farm (Les Trinottières, France). During this experimentation, several plant (silage) and animal products (blood, intestinal chime, feces) were collected and stored (−20 °C) for further analyses.

In a previous paper (*12*) we have reported the development of sensitive detection methods targeting numerous oligo- and multicopies DNA sequences. These quantitative real-time PCR sensitive methods, which comply with ISO DIS standards by their ability to identify amplicons, should discard any false positive or negative results and should provide very sensitive results by targeting oligo- and multicopy systems in 2 cellular compartments: the nucleus and a plastid. An immunological method was also developed, as well as DNA and protein extraction methods (*12*).

Here, we report the results of PCR and ELISA analyses performed on blood samples collected during the experiment carried out at "Les Trinottières" in 1998, to search for the presence of plant DNA (endogenous and transgenic) and protein (Cry1A(b)) in the blood of cows fed with silage of either conventional or GM (Bt176) corn.

MATERIALS AND METHODS

Corn Silage. Conventional (Rh208 cultivar) and Bt176 genetically modified (Rh208 Bt) corn hybrids were grown and harvested to yield silage according to standard farming practices, as previously described (*11*).

Dairy Cows. *Experimental Design.* Two sets of 24 Holstein cows (7 primiparous and 17 multiparous) yielding about 8,500 kg of milk per year were used. Animals were paired and assigned to each experimental diet before calving (*11*). All cows first received the same diet from week 2 before calving to week 9 post calving (*11*). Then, cows were individually fed with daily experimental 70% corn silage diets (conventional or GM hybrid) complemented with 28.5% concentrates, for 13 weeks using Calan-type feeding doors.

Management and Care. The experiment was performed according to INRA rules of animal care (*13*), thus taking care of the welfare of the animals. During and after the experiments, cow health was followed through observations of the animal behavior, twice a day and during feeding and milking, for possible abnormal individual or within-herd signs. Cows were weighed at the beginning and end of experiments, and every 28 days during the Trinottières experiment. Health follow-up of each animal included records of each individual event and medical treatments.

Blood Sampling (Collection, Storage and Processing). Cows ($n = 48$) were sampled for several matrices (feces, rumen juice, blood, milk) taking care for minimizing external contamination (*11*). Blood (10 mL) collected in two 5 mL standard glass tubes with Li-heparin was stored at −20 °C until analysis. Ten cows were randomly selected in each feeding group (conventional and GM) according to a statistics based experimental design.

DNA Preparation. DNA was extracted in 3 independent triplicates from total blood samples (2.5 mL per extraction) and quantified by fluorimetry as described by Petit et al. (*12*). Bovine and corn haploid equivalent genomes were assumed to be 3.3 and 2.5 pg DNA, respectively (*14, 15*).

5′-Nuclease PCR (TaqMan) Assays. Detection of transgenic sequences was carried out as previously described (*12*). Amplicon sizes ranged between 88 and 211 bp to overcome possible DNA degradation. GMO targets were 35S promoter (p35S) and Bt176-specific junction sequence. Plant targets were the monocopy maize-specific sequence (*adh* promoter) and two highly sensitive multicopy sequences from plant nucleus (26S rRNA gene) and chloroplast (*psaB* gene). The copy numbers of 26S rRNA gene and of chloroplast genome, relative to nuclear haploid genome, were assumed to range from 2,500 to 5,000 (*16*) and 500 to 50,000 (*17*), respectively. The positive control targets a monocopy mammalian gene encoding α_{s1}-casein (*CSN1S1*), a major milk protein.

Primer pairs and internal fluorogenic probes (*12*) were purchased from Applied Biosystems (Courtaboeuf, France).

PCR Conditions. Amplification reactions were run in triplicate on each DNA sample either on an ABI Prism SDS 7700 or on an ABI

Prism SDS 7900 Instrument (Applied Biosystems, Foster City, CA) under standard conditions (*12*). Reactions were performed in a 50 μL total volume containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems). Following previous assay optimization (*12*), primer and probe concentrations were 300 nM, except for 26SUV1/26SUV2 (900 nM), and 200 nM, respectively. Ten microliters of DNA samples (nondiluted and 1/10 diluted for the first series of analyses; 1/10, 1/20 and 1/50 diluted for the second series) were used as template. Nontemplate controls (NTC) were included in each series.

Cry1A(b) Protein Detection by ELISA. Three aliquots (100 μL) of each blood serum sample were analyzed using a commercially available double-antibody sandwich (DAS)-ELISA kit (Agdia Inc., Elkhart, IN), following manufacturer's instructions. A standard curve (0.1 to 10 ng/mL) was prepared using blood serum samples spiked with purified, Cry1A(b) protein dissolved in water (**Figure 1**). The mean absorbance (450 nm) was calculated and used as described (*12*). Positive (PC) and negative (NC) Cry1A(b) controls were prepared, as well as a serum positive control (SPC), spiking the serum from a conventional silage fed cow (# 1016) with the kit Cry1A(b) positive control solution diluted 1/4.

Data Analyses. DNA was extracted from blood samples, in triplicate in two laboratories (AFSSA and INRA Jouy-en-Josas). Blood samples were analyzed for 10 cows per feeding to minimize analysis costs meanwhile ensuring the yield of statistically reliable data. Inhibition effects are characterized by points which appear above and on the right of the linear regression of the Ct vs the logarithm of the DNA concentration. These points are outliers of the linear regression and can thus be easily identified (*18*). Correlations between nondiluted, 1/10, 1/20 and 1/50 diluted samples were computed to investigate laboratory and apparatus effects.

RESULTS

DNA Detection. The concentration of DNA extracts ranged between 7.5 and 161 ng/μL. DNA integrity was checked by agarose gel electrophoresis on randomly chosen DNA samples: high molecular weight DNA fragments (more than 20 kb) were observed for all samples (data not shown).

First Set of PCR Analyses. In a first experimental set, 4 (p35S, *adh*, *psaB*, *CSN1S1)* qRT-PCR systems previously developed and validated (*12*, *19*, CRL-GMFF validated methods http://crl-gmo.jrc.it/) were used to detect GMO and plant DNA sequences among animal DNA. Ct values lower than 45 cycles were considered as positive results. The strong constitutive promoter p35S was used, since it is considered as one of the most sensitive validated PCR tests for GMO plant screening, including Bt176. *Adh* and *psaB* were used as models of nuclear oligocopy system and chloroplastic multicopy targets, respectively. Finally, *CSN1S1* was used as a positive internal amplification control.

Given the extreme sensitivity of the PCR assays, analyses were carried out using extensive precautions to avoid as much as possible laboratory-derived sample contamination during the course of the experiments (blood handling, DNA extraction and quantitation, PCR).

All samples were *CSN1S1* tested positive, with an inhibitory effect for nondiluted DNA samples which was significantly reduced after 1/10 dilution (**Figure 2**). Accordingly, the results presented below are relative to the 1/10 dilution.

The PCR assay targeting the 35S promoter gave negative results for all bovine DNA samples with the exception of 2 DNAs, from cow # 278 (fed with Bt176) and cow # 282 (fed with CCS), both tested positive with Ct between 34 and 41 (**Table 1**). DNA was extracted in triplicate from cow blood samples. Since blood samples from cows # 278 (Bt176) and # 282 (CCS) were extracted the same day in parallel on the same site, one cannot exclude an oversight pooling of DNA tubes to explain the positive result recorded with the 35S promoter

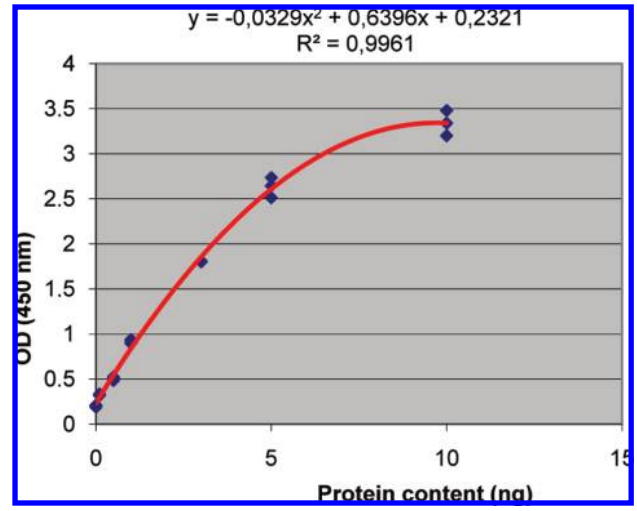


Figure 1. Regression (polynomial) curve obtained using the Agdia sandwich-ELISA test from a bovine blood serum, spiked with increasing amounts (0.1 to 10 ng/mL) of protein Cry1A(b).

system for the CCS-fed cow (# 282). To test this hypothesis, we checked the origin of blood DNA. A multilocus genotyping test based on the 9-microsatellite ISAG reference panel (http://www.isag.org.uk/ISAG/all/02_PVpanels_LPCGH.doc) and developed for individual identification and parentage testing (Labogena, France) was used for this purpose, to compare haplotypes from DNA used for PCR tests and DNA re-extracted from blood samples. Meanwhile, no CaMV contamination was observed for these 2 samples (data not shown) by the CaMV control test.

On the other hand the *adh* targeting assay is considered as negative even though 7% of the PCR points tested randomly positive ($Ct < 45$). Nevertheless, it is worth noting that cow # 282 is again weakly positive ($42.5 < Ct < 47.5$) in 2 of the 3 testing laboratories. Given the similar number of copies ($n = 2$) per diploid genome, the discrepancy observed between *adh* and p35S results could be explained by the relative PCR efficiency of p35S and *adh* tests.

From the *CSN1S1* set of data (**Figure 2**), it appears that the 1/10 dilution suppressed most of the inhibitory effect which was completely removed at the 1/50 dilution, as demonstrated by the good compliance between DNA contents measured by OD_{260nm} and *CSN1S1* qRT-PCR.

The sensitive chloroplast *psaB* multicopy PCR test has been performed to circumvent a possible lack of detection resulting from a low sensitivity of oligocopies targeting PCR tests. With 93% of PCR points weakly positive (Ct between 34 and 49, mean $= 41.5 \pm 3.1$), the *psaB* multicopy target was detected in most of the samples. This *psaB* amplification is very likely due to a slight residual presence of plant DNA in blood rather than to unspecific amplifications, since the sequence specificity of *psaB* test was previously checked in silico. Those results are consistent with previous reports (*3*) in few samples of several species.

These results thus confirm the ability of chloroplastic DNA to cross the bovine intestinal barrier. Since the *psaB* target might be more protected from DNA degradation by the chloroplastic membrane, and therefore more easily detected, it might be of interest to address the fate of nuclear DNA by targeting nuclear multicopies.

Second Set of PCR Analyses. A second set of PCR tests was performed on dilutions 1/10, 1/20 and 1/50 (to avoid any inhibitory effect) with a more specific GMO test (Bt176) and a nuclear (26S rRNA) multicopy plant test (*12*).

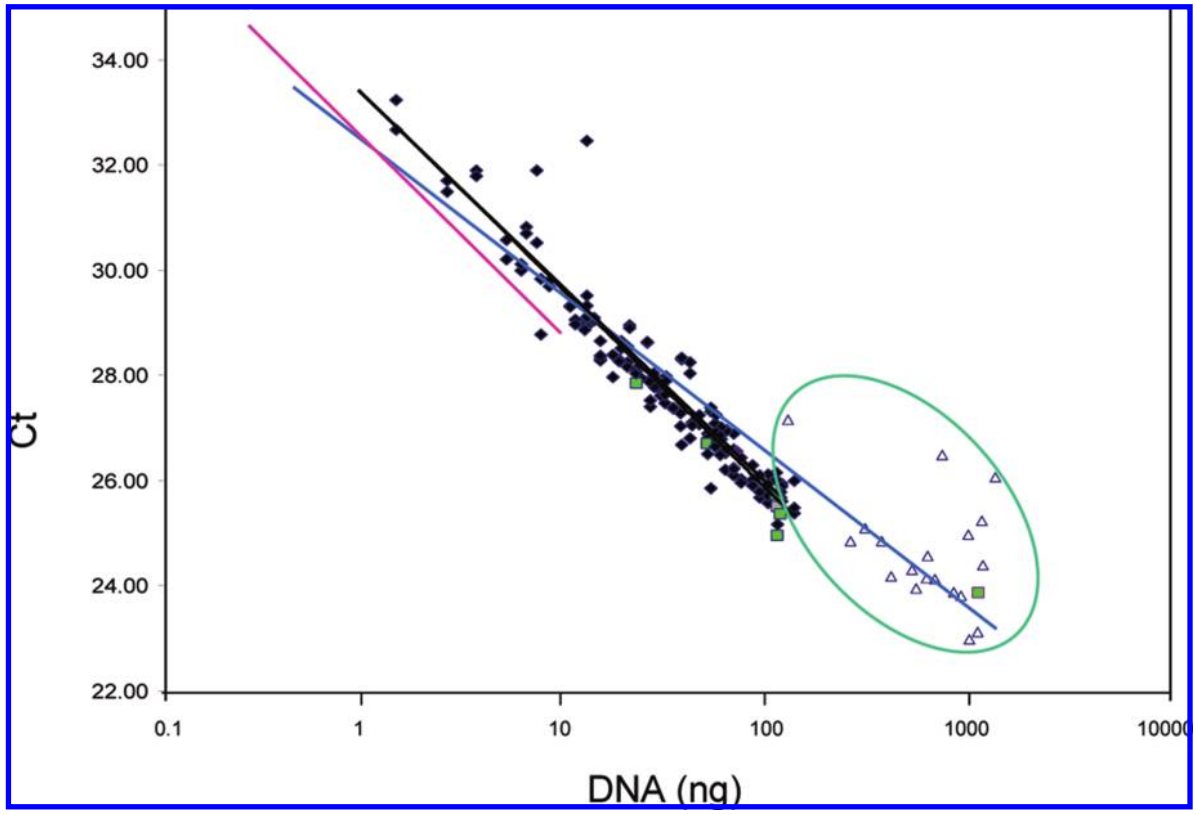


Figure 2. Correlation between the amount of DNA (ng), determined spectrophotometrically, and the cycle thresholds (Ct) of quantitative real time PCR experiments determined for the CSN1S1 system. All the PCR points yielded during the first and the second set of experiments were taken into account (blue regression line: $y = -1.3021$ Log $x + 32.29$). When undiluted DNA samples (circled white triangles, first set of experiments) are discarded, regression analysis gives the black regression line ($y = -1.6253$ Log $x + 33.29$) which is parallel to the pink standard's regression curve ($y = -1.7336$ Log $x + 32.67$). The green squares correspond to cow # 278.

All samples were tested negative with Bt176 sequences, including the two p35S positive samples reported in the first experiment set. Since Bt176 copy number is half of p35S, we cannot discard the hypothesis of GMO derived sequence being present in these 2 cow blood samples at the limit of detection level. In such a case, the discrepancy between p35S and Bt176 might be due to differences between PCR test efficiencies as previously observed (*12*).

Only twenty percent of PCR points were positive with the 26S rRNA but with an average Ct lower than that observed for *psaB* in the first set of experiments. However, the curves' aspects look unusual (data not shown) when compared to the standard curves. Since the primers and probe integrities and efficiencies were positively assessed on plant DNA, we can assume the curves' aspects are related to either an inhibition and/or the degradation of the targeted nuclear DNA.

In conclusion, both sets of experiments only show faint amounts of plant DNA in the blood of cows fed with GM corn silage (Bt176) or with conventional corn silage (CCS).

Cry1A(b) Protein Detection. Only one serum (cow # 1033) of twelve GM corn silage fed cows was positive. Conversely, all the blood serum samples from conventional silage fed cows were negative (**Figure 3**). It should be noted, however, that this cow (# 1033) did not give any positive PCR result. In the other hand, cows # 282 (CCS) and # 278 (Bt176) which were positive with p35S were both found negative with ELISA. Even though it has been shown that the ensiling process markedly decreases the presence of full size Cry1A(b) protein, more than 20% of the initial Cry1A(b) protein was still detectable after 61 days (*20*). Therefore one cannot rule out the possibility for Cry1A(b) to be transferred through the intestinal barrier and found in blood. We can also speculate that the unique positive result recorded might be due to a contamination during sample collection, preparation or analysis.

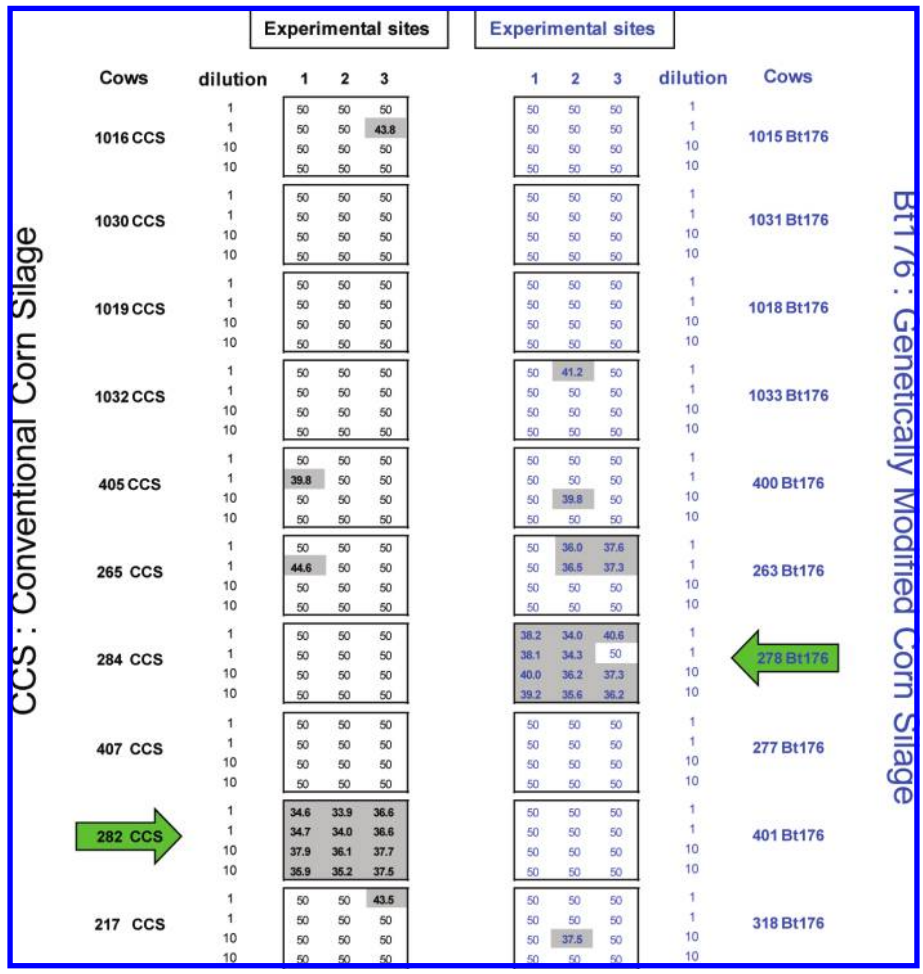
DISCUSSION

The introduction of commercial GM crops during this past decade raised consumers' concerns about the fate of GMOs and their derived products into food chains. In order to meet consumers' requests, labeling of foodstuffs beyond a threshold of fortuitous presence of GMOs has been made mandatory in several countries. Detection methods have been developed for control purposes using proteins and mostly DNA as analytes.

The request of consumers for transparent information and traceability induced the further labeling of feedstuffs. The labeling of products derived from animal fed GMOs was not required in any country (*1*). This decision came from the presumption that no analyte could be found in animals. No experimental data supported this presumption, and the purpose of the present research was to investigate the availability of transgenic material in animal blood. It was most probably anticipated that, if any analyte could ever be detected, it would be at analytical costs completely disproportionate to the putative risk, should the methodology be used for routine testing purposes. However, developing new detection methodologies for low GMO-derived analytes' concentrations could prove to be helpful for pedagogic and litigation purposes.

The first studies investigating the fate of plant DNA in ruminants took place more than three decades ago and showed that most plant DNA, whether fed as a naked molecule or as whole plant derived product, is quickly degraded to oligonucle-

Table 1. Real-Time PCR Analysis for the Detection of p35S Sequence in DNA Extracted from Blood Samples[a]



[a] Ct values below 45 (positive) are shaded in gray. CCS cows were conventional corn silage fed cows, whereas Bt176 cows (blue) were fed with silage from GM corn. Each DNA sample was analyzed in duplicate, at two dilutions (1/1 and 1/10), on three sites (1, 2, 3). One cow in each group (green arrows) was tested positive, whatever the dilution and the site were.

otides, nucleosides and bases throughout the digestive tract (*3, 20, 21*). However, concomitantly with the emerging of new and sensitive detection methods such as PCR, the results published on phage M13 sequence in mice (*7*) relaunched the polemics. A knowledge on the fate of DNA and proteins in the products derived from animals fed with transgenic crops raised is of scientific interest (*3*).

Since then, numerous reports have attempted to describe the persistence of transgenes. So far published papers focused on mice, chickens, pigs, salmons fed among others PCR amplicons, phage and plants products, with data on specific tissues such as liver, meat and derived body fluids such as milk and blood (*3*). Most of the accumulated data (*3*) converge toward the detection of high copy number sequences (e.g., from plastids) but not of GMOs.

In an attempt to set up a more systemic approach, we developed a complete set of well-characterized detection methods complying with the ISO DIS 24276 and 21570 standards (*12*). These methods address animal (α_{s1}-casein), plant (*ADH* promoter) and GMO (35S promoter and Bt176 specific junction sequence) sequences, some of them having an exquisite sensitivity as being nuclear (26S rRNA gene) or chloroplastic (*psaB* gene) multicopy targets. Since blood is a vector between the digestive tract and animal matrices, the presence or absence of DNA and/or protein in blood would be a good marker of their putative presence in other animal matrices.

Our study shows the ability of detecting rather low contents of plant nuclear and chloroplast DNA fragments which entered, at least temporarily, the bovine blood circulation. A similar situation was observed for salmon (*5*). Maize nuclear DNA, both mono- and multicopy sequences, was less detected than chloroplastic DNA, probably due to its lower number of copies but also probably because of a greater sensitivity to degradation. These results are in line with the higher number of chloroplastic targets compared to the nuclear ones (*17*) and results previously reported by other authors. Indeed, the chloroplastic DNA target might be more protected from DNA degradation by the chloroplastic membrane than nucleus targets are by the nuclear membrane. Whatever the cause of such a better detection of chloroplastic sequences, the PCR detection methods described in our papers should be usable for tracing the future chloroplast transformed GMOs, even the GMO content will not be quantifiable by the current tools.

Our data are in accordance with some recent studies which have already mentioned the detection of plant nuclear and/or chloroplast sequences in several animal organs and tissues (*3*−*5*). In addition to pathogenic bacteria and viruses, plant DNA fragments and very likely DNA from nonpathogenic microorganisms and animals can under appropriate conditions cross the ruminant intestinal barrier and stay for a more or less long period in blood before clearing. Despite the fact that our data

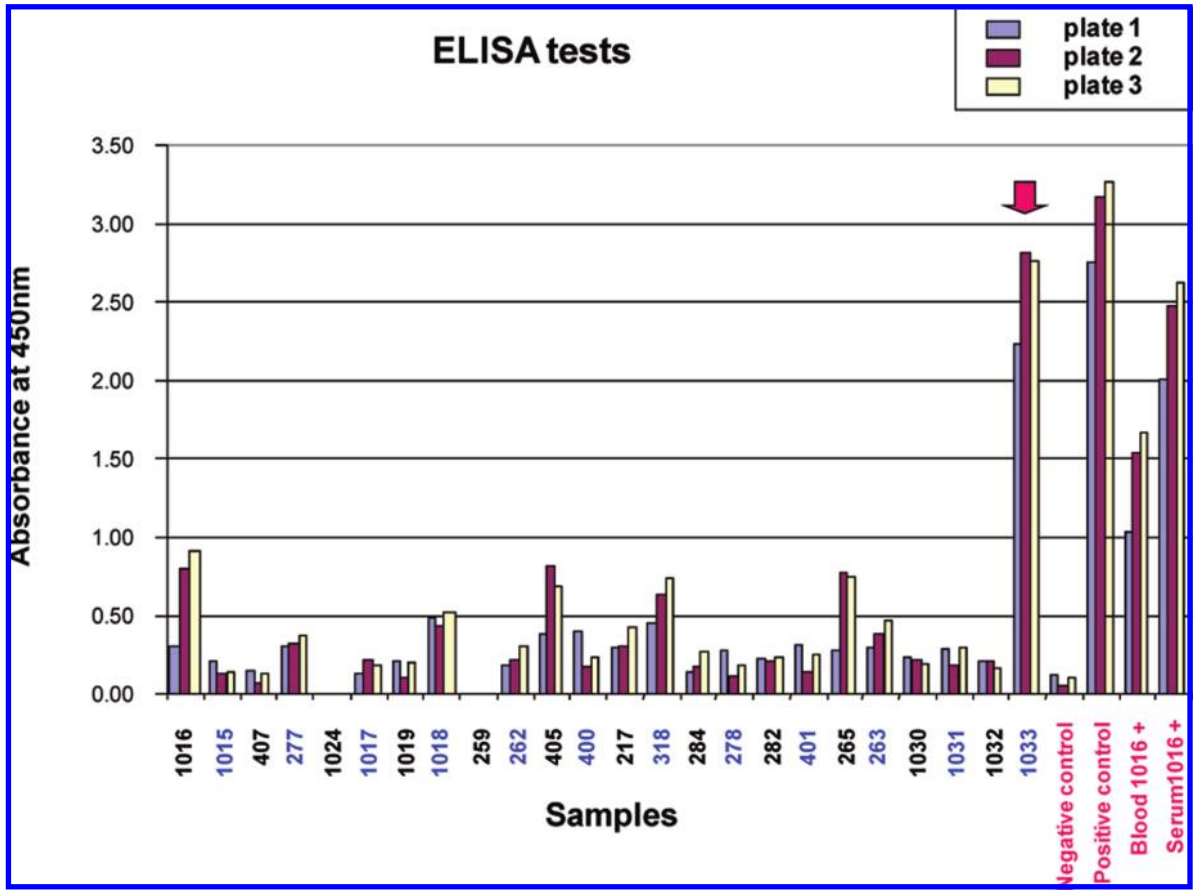


Figure 3. ELISA tests. All blood samples were determined in triplicate, and the analyses were repeated 3 times (3 plates). All samples giving an absorbance higher than 1.0 at 450 nm with the 3 plates were considered as positive. The only positive sample (cow # 1033, red arrow) was fed with Bt176 corn silage. Cows fed with conventional corn silage (CCS) are numbered in black; cows fed with Bt176 corn silage are numbered in blue. Blood and serum samples from cow # 1016 were spiked with 2.5 ng of protein Cry1A(b).

confirm the ability of small plant DNA fragments (*psaB* and 26S rRNA) to cross the intestinal barrier, we were unable to observe the presence of transgenic DNA sequences (35S promoter and Bt176 specific junction sequence) and protein in bovine blood. As far as we know, only one study reported presence of a *CryIA*(b) DNA sequence in blood (*4*).

According to our ability to detect only multicopy DNA targets, we can expect that GMO originating sequences (35S promoter and Bt176 construct specific), whose copy number is similar to the undetectable *ADH* corn reference gene, might be however found but only at very low levels in cow blood. The slightly positive results obtained with some samples were probably due to contamination during the sampling step and can be considered as an artifact as they have not been confirmed by the different methods used. This assumption is supported by a further DNA extraction from sample # 282 whose p35S analysis was then negative. Unfortunately, a second DNA extraction from sample # 278 was not possible due to the complete use of the blood sample.

Given the methods' LOD (multicopy target methods being several hundreds to 1,000 more sensitive than the oligo-copy target methods whose LOD is almost 20 copies (*12*)) and volume analyzed per PCR (5 μL of test portion corresponding to 125 μL of blood), GMO sequences might be found only when using at least 1,000-fold larger blood sample assuming the mean number of GMO sequence present per tube is at least of 5, i.e. more than 125 mL (see Appendix). Such an approach would be technically and economically unrealistic owing to the volume to be treated and the DNA purity to be reached unless using more performing new forthcoming DNA extraction and purification and/or detection methods.

While these results are quite new in the livestock production area, the existence of extracellular nucleic acids in the human circulation was first reported by Mandel and Metais in 1948 (*22*). They demonstrated that both DNA and RNA could be detected in the plasma of sick as well as healthy individuals. To date, circulating cell-free nucleic acids analysis has been studied in many clinical scenarios, for example, cancer detection (*23*), prenatal diagnosis (*24*), and monitoring of both trauma patients and organ transplant recipients (*23*, *25*). This circulating DNA is a prognosis tool which is increasingly studied (*26*). Circulating DNA has also been detected in nonpathological situation (*23*, *27*, *28*). Moreover, circulating mRNA and DNA in human blood and plants have also been reported and seem to be acting as cell-to-cell messengers (*23*). Therefore, it was highly foreseeable to find DNA originating from feed in cows' blood. The very low level of foreign DNA found is probably due to a daily clearance by DNases present in blood as observed in humans (*29*).

DNA is a new way for animal vaccination but is also reported as being involved in mechanisms linked to allergies (*30*). DNA vaccines require carriers to improve their stability and persistence. The occurrence of persistent plant nuclear and chloroplastic DNA sequences in blood (ref *3* and this paper) might raise therapeutic hopes but at the same time new concerns on safety issues, even though the circulation of foreign DNA is occurring since the evolution of circulatory systems. Bacteria could be vehicles for this vaccination purpose, but stability of

carrying plasmids has to be improved for successful application (*31*). Since chloroplast membranes probably protect its DNA, GM chloroplasts might play this carrier role of vaccinating plasmids in the future. Consequently, one can expect that adsorption and nucleus integration of foreign circulating DNA, as reported in some papers (*32*, *33*), will constitute a growing research area, especially in the case of chloroplast transformation (*34*).

Similarly to previous observations of other authors (for a review see ref *2*), Cry1A(b) was not detectable with our immunological tools in the bovine blood samples. As previously reported (*2*), the *Bt* protein almost disappears during the ensiling process (*20*) and could not be detected in the silage (*35*). However, Einspanier et al. (see ref *3*) as well as Lutz et al. (see ref *3*) have detected Cry1A(b) fragments in the gastrointestinal tract of cows fed with Bt176 silage. Accordingly, the lack of detection in our experiments can be due to a low content of Cry1A(b) protein, as Bt176 is less Cry1A(b) producing than other GMOs (*36*). A way to conclude might be to search for anti-Cry1A(b) antibodies in sera, provided pure Cry1A(b) is available. Additionally, the detection of GM DNA and/or protein might depend on a window of uptake as previously observed for salmon (*5*).

In conclusion, our study indicates, as previously reported (*3*), that it is almost economically impossible to reliably trace, with the current detection methodologies, dairy cows fed with present genetically modified plants. Therefore, the consumers' demand for information and traceability can only be met with a documentary traceability system given the economic and technical difficulties of ensuring a proper analytical control of labeling.

ABBREVIATIONS USED

GMO, genetically modified organism; GM, genetically modified; PCR, polymerase chain reaction; GIT, gastrointestinal tract; NTC, Non Template Control; ELISA, enzyme linked immunosorbent assay; CCS, conventional corn silage.

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APPENDIX

Assume that the mean number of GMO sequence per PCR tube is λ and that the LOD is greater than 20; then the probability to detect GMO in at least one of *n*PCR tubes is

$$1 - P^n$$

where P is the probability of having fewer than 20 GMO sequences in one tube, that is

$$P = \sum_{i=0}^{20} e^{-\lambda} \frac{\lambda^i}{!i}$$

The array below gives the probability for some values of λ and *n*:

λ =	5	10	15	20
n				
10	0	0.0158	0.5794	0.997
100	0	0.147	0.9998	1
1000	0.0001	0.796	1	1

Therefore, there is almost no chance to get a positive result even with 1,000 PCR tubes if the number of GMO sequences per tube is only about 5 copies. The mean number of GMO sequences per tube must reach 15 to have a chance of detecting in 1 out of 100 PCR tubes, and must reach 20 to detect in 1 out of 10 tubes. Note that DNA extracted from 2.5 mL of blood was resuspended into 200 μL, which allow to fill in 40 tubes of PCR (5 μL per tube). Thus, if the mean number of GMO sequences per tube reaches 15, so that 100 PCR tubes are required to get a good chance ($P = 0.9998$) of detecting the GMO sequence in at least one tube, the DNA extraction must be carried out from 6.25 mL to fill in the 100 needed PCR tubes.

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