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Detection of genetically modified DNA sequences in milk from The Italian market

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Abstract

The possible transfer and accumulation of novel DNA and/or proteins in food for human consumption derived from animals receiving genetically modified (GM) feed is at present the object of scientific dispute. A number of studies failed to identify GM DNA in milk, meat, or eggs derived from livestock receiving GM feed ingredients. The present study was performed in order to: (i) develop a valid protocol by PCR and multicomponent analysis for the detection of specific DNA sequences in milk, focused on GM maize and GM soybean; (ii) assess the stability of transgenic DNA after pasteurization treatment and (iii) determine the presence of GM DNA sequences in milk samples collected from the Italian market. Results from the screening of 60 samples of 12 different milk brands demonstrated the presence of GM maize sequences in 15 (25%) and of GM soybean sequences in 7 samples (11.7%). Our screening methodology shows a very high sensitivity and the use of an automatic identification of the amplified products increases its specificity and reliability.

Moreover, we demonstrated that the pasteurization process is not able to degrade the DNA sequences in spiked milk samples. The detection of GM DNA in milk can be interpreted as an indicator of fecal or airborne contamination, respectively, with feed DNA or feed particles, although an alternative source of contamination, possibly recognizable in the natural environment can be suggested. Further studies, performed on a larger number of milk samples, are needed to understand the likely source of contamination of milk collected from the Italian market.
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Keywords: Genetically modified DNA; Milk; PCR

Introduction

Safety assessment and risk management of genetically modified (GM) food, not only takes into account the real risk, objectively determined according to epidemiological criteria, but also the perceived risk, not necessarily demonstrated but hypothesized and only assumed as such. At present, the USA and Canada (82%) and Argentina (17%) grow the vast majority of GM crops but China, Australia, South Africa, Mexico, Spain, France, Portugal, Romania and the Ukraine are also growing GM crops (James, 2000; Phipps et al., 2002) that may be used in diets for productive livestock.

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The possible transfer and accumulation of novel DNA and/or proteins in food for human consumption derived from animals receiving GM feed is at present the object of scientific dispute (Beever and Phipps, 2001; Phipps et al., 2002, 2003). Any investigation into whether modified DNA or novel proteins consumed by animals have the potential to affect animal health, or to enter the food chain, should consider the fate of these molecules within the animal and should put into context the actual amounts of DNA entering the digestive system of a typical dairy cow. Dairy cows can consume 24 kg of dry matter per day. If a dairy cow's ration includes 60% GM maize (as silage or grain) per day, then that cow is eating approximately 60 grams of total DNA a day, 54 micrograms of which is transgenic DNA (Beever and Phipps, 2001).

Milk is an important food due to its nutritional value, for its population usage, and also for its preferential use by vulnerable subjects, such as babies, children, the elderly. The dairy cows consume and process large quantities of raw materials and feed, producing a large volume of high-quality milk. Environmental conditions may influence the intake of the nutrients, including GM molecules, and their transfer through the digestive system and the blood circle to the mammary gland (Beever and Phipps, 2001; Poms et al., 2003; Mc Allan, 1982; Schubbert et al., 1997). Moreover, due to the great stability of genomic DNA, sterilization or pasteurization could not be effective for the full degradation of the molecule that, therefore, could maintain full functional integrity.

A number of studies have now been conducted in which GM DNA has not been detected in milk, meat, or eggs derived from livestock receiving GM feed ingredients (Phipps et al., 2002, 2003; Faust, 2000; Flachowsky and Aulrich, 2001). Moreover, two studies determined the presence of GM DNA in different parts of the ruminant digestive tract (Phipps et al., 2003; Faust, 2000).

The present study was performed in order to:

(i) develop a valid protocol for the detection of specific DNA sequences in milk, focused on GM maize (Maximizer maize) and GM soybean (RoundUp Ready Soybeans), by PCR and multicomponent analysis (MA);
(ii) assess the stability of transgenic DNA after the pasteurization treatment of spiked milk samples;
(iii) determine the presence of GM DNA sequences in milk samples collected from the Italian market.

Materials and methods

Reference standards and DNA extraction

Transgenic soybean and maize reference standards (Fluka, Buchs, Switzerland) were used. These standards are Certified Reference Material consisting of dried soybean and maize powders and were developed by the Institute for Reference Materials and Measurements (IRMM). DNA was extracted from reference standards using NucleoSpin Food kit (Macherey-Nagel GmbH& Co. KG, Germany), in conformity with the protocol suggested by the producer, and following microspectrophotometric quantification by GeneQuant proRNA/DNA Calculator (Amersham Pharmacia Biotech, Italy).

Polymerase chain reaction amplification

The primers labeled with the fluorescent reporter dye on the 5′ end were described by Vaitilingom et al. (1999). For both GM DNA, two PCR systems were chosen: one for the total detection of maize or soybean (EM endogenous maize, ES endogenous soybean), and the other for the specific detection of "Maximizer" maize or "Roundup Ready" soybean (TM transgenic maize, TS transgenic soybean). The EM PCR system amplified a sequence of 10-kDa *zein* gene, encoding a methionine-rich 10-kDa zein protein from maize, and the TM PCR system targeted the *CryIA(b)* gene, encoding the CRYIA(b) protein (Bt-delta-endotoxin) from *Bacillus thuringiensis* (Vaitilingom et al., 1999). The ES PCR system was developed on the *Le 1 lectin* gene *le1,* encoding soy lectin, and the TS PCR system on the CP4 *EPSPS* gene, encoding the synthetic enzyme 5-enol-pyruvylshikimate-3-phosphate synthase derived from *Agrobacterium* sp. strain CP4 (Watrud et al., 2004). For endogenous maize, primers were labeled with 6-carboxyfluorescein (FAM); for transgenic maize, primers were labeled with rhodamine (RHOD); for endogenous soybean, primers were labeled with tetrachloro-6-carboxyfluorescein (TET); for transgenic soybean, primers were labeled with 6-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein (HEX). Each of the fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM 310 instrument, the fluorescent signals are separated by a diffraction grating according to their wavelengths and projected onto a CCD camera in a predictably spaced pattern. Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the four dyes. The goal of MA is to isolate the signal from each dye so that for example signals from 5′-FAM labeled products are displayed in the electropherogram for blue, but not in those for green, yellow or red. Amplification reactions (25 μl) were performed with 1.5 units of Taq DNA polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 mM Tris-HCl, 12.5–25 pM of each primer and 1–5 μl of DNA. PCR reactions were run on a Mastercycler Gradient (Eppendorf) under the following condition: 3 min at 94 °C, and then 20 s at 94 °C, 40 s at 62 °C, 1 min

at 72 °C, for 40 cycles and 3 min at 72 °C. Amplification products were visualized after gel electrophoresis (GE) using precast agarose minigels at 4%, in TBE 1X (Reliant gel system, BioWhittaker Molecular Applications, Rockland, ME USA), and photographed. In every PCR run, positive and negative controls were included to ensure reproducibility and absence of contaminants. For positive control, reference DNA consisting of purified maize and soybean DNA, was amplified in parallel with the samples to ensure the correct performance of the PCR; for negative control (buffer blank), water instead of DNA was added to the PCR mix to check for cross contamination with maize or soybean DNA in the PCR mix or its constituents (Klaften et al., 2004). Only consistent results were considered throughout the study, that is gel showing only the correct result in control tests.

To 1 μl of the amplified sample 25 μl of formamide molecular weight marker (ROX 500) was added. Samples were subjected to capillary electrophoresis using an automatic analyzer (ABI PRISM 310 Genetic Analyzer, PE Biosystem) and specific identification of amplified products was performed by the GeneScan Analysis Software (PE Biosystem). Positive and negative controls were subjected to each assay and only consistent results were considered throughout the study, that is assays showing only the correct result in control tests.

Identification of endogenous and transgenic sequences of maize and soybean in milk samples before and after pasteurization

In order to assess the sensitivity and specificity of our PCR detection method, known amounts of endogenous and transgenic DNA (respectively, range 10–1000 ng and 12.5–100 ng) were added to 200 μl of whole fresh milk samples of different brands from the Italian market. After DNA extraction and spectrophotometric quantification, followed by PCR and GE, the critical minimum DNA for successful PCR analysis (LOD, limit of detection) was determined. Moreover, in order to assess the stability of transgenic DNA after pasteurization treatment, milk samples containing GM DNA were incubated at 72 °C for 20 s, and DNA was extracted, quantified and amplified as described above. Amplification products were visualized after GE and MA, the capillary electrophoresis of amplified products by an automatic analyzer, which allows the separation of the different fluorescent dyes in several spectral components, to identify the amplified products in a specific way and to exactly determine their molecular weight.

Identification of endogenous and transgenic sequences in milk samples

A total of 59 cow milk samples of 12 different brands purchased in several retail outlets in Catania, Sicily, Italy were tested for the presence of the transgenic sequences of maize and soybeans. Moreover, one sheep milk sample was included, from a private producer. Furthermore, five representative samples of the different milk brands were screened for the presence of the endogenous sequences of maize (*zein*) and soybeans (*Le1 lectin*). The identification of endogenous and transgenic DNA was performed as described above in duplicate. Positive detection of transgenic DNA was accepted if duplicate PCR tests showed the correct amplicon based on MA, while negative detection of transgenic DNA was accepted if no amplicon was obtained based on MA.

Results

Identification of endogenous and transgenic sequences of maize and soybean in milk samples before and after pasteurization and LOD determination

In order to assess the sensitivity and specificity of our PCR detection method, DNA obtained from milk samples of three different brands from the Italian market each spiked with known amounts of endogenous and transgenic reference maize and soybean DNA were analyzed in duplicate by PCR and GE: the fragment of the expected size was correctly and reproducibly amplified in all spiked samples: A total of 69 bp for endogenous maize; 80 bp for endogenous soybean; 106 bp for GM maize; and 145 bp for GM soybean (Fig. 1). MA showed a fluorescence peak where the amplified fragment of the expected dimension was present (Fig. 2). Moreover, it was possible to distinguish the amplification product from extra artifact bands by reading the fluorescent marker. The spiking experiments (200 μl milk), found that $\geqslant 0.5$ ng of DNA with 2% of GM DNA could be reproducibly and repeatedly detected. Therefore, GM DNA final LOD accounted for $\geqslant 10$ pg (200 μl of milk).

Moreover, in order to assess the stability of transgenic DNA after pasteurization treatment, fresh milk samples were subjected to conditions that simulate pasteurization, and then analyzed by PCR and MA, producing amplified fragments of correct size for both primer systems (Fig. 1).

Identification of endogenous and transgenic sequences in milk

A total of 60 milk samples were analyzed for transgenic sequences in duplicate assays: A total of 28 were normal milk, 14 were organic milk, 11 were milk labeled as "addressed to children", that is whole sterilized cow milk supplemented with minerals and

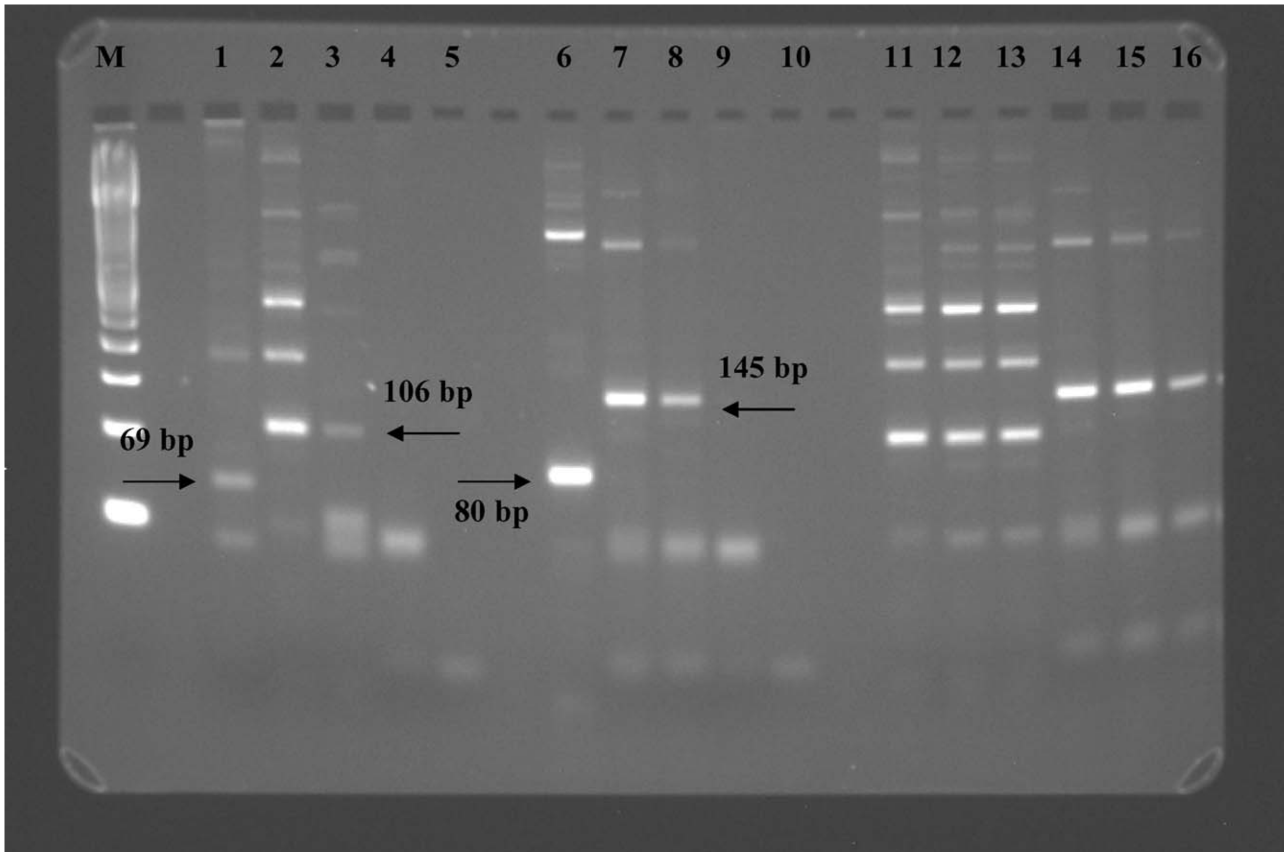

Fig. 1. Detection of transgenic DNA in milk sample. Lane M: DNA size marker 50 bp; Lane 1: spiked milk sample D3 with DNA from reference standard maize amplified with endogenous primers; Lane 2: spiked milk sample B1 with DNA from reference standard GM maize amplified with transgenic primers; Lane 3: milk sample C1 from the Italian market amplified with transgenic primers; Lane 4: milk sample D2 from the Italian market which failed to give any amplification with transgenic primers; Lane 5: buffer blank; Lane 6: spiked milk sample B1 with DNA from reference standard soybean and amplified with endogenous primers; Lane 7: spiked milk sample B1 with DNA from reference standard GM soybean and amplified with transgenic primers; Lane 8: milk sample A1 from the Italian market amplified with transgenic primers; Lane 9: milk sample D2 from the Italian market which failed to give any amplification with transgenic primers; Lane 10: buffer blank; Lane 11: spiked milk sample B1 with DNA from reference standard GM maize and amplified with transgenic primers; Lane 12 and 13: spiked milk samples B1 with DNA from reference standard GM maize amplified with transgenic DNA after the pasteurization treatment; Lane 14: spiked milk sample B1 with DNA from reference standard GM soybean and amplified with transgenic DNA; Lane 15 and 16: spiked milk samples B1 with DNA from reference standard GM soybean amplified with transgenic DNA after the pasteurization treatment.

vitamins, six were organic milk labeled "addressed to children", and one was raw sheep milk from a private farm. After PCR and GE analysis, the presence of the transgenic sequences of maize was shown in 23 samples (38.3%): A total of 13 were normal milk samples, six organic milk samples, two milk addressed to children and two organic milk addressed to children. The presence of the transgenic sequences of soybean was shown in a total of seven samples (11.7%): Five were normal milk samples and two organic milk samples. Of the 20 organic milk samples six were positive for GM maize (30.0%) and two for GM soybean (10.0%). Three samples from the same company were positive for transgenic sequences of both maize and soybean. Duplicate assays on the same sample as well as different samples from the same milk brand systematically showed consistent results. MA confirmed 15 positive samples for transgenic sequences of maize (25.0%) and all the seven positive samples for the transgenic sequences of soybean (11.7%) (Table 1). Therefore, for the transgenic sequences of soybean the agreement of the two tests is 100%. Considering MA as the gold standard test, due to the controlled conditions of automatic analysis, for the transgenic sequences of maize the sensitivity of GE is equal to 93.3%, its specificity is 80% and its accuracy is 83.3%. Furthermore, five representative samples of the different milk brands were screened for the presence of the endogenous sequences of maize (*zein*) and soybeans (*Le1 lectin*). Particularly, two samples were positive for the endogenous sequences of maize (40.0%) and three samples were positive for the endogenous sequences of soybeans

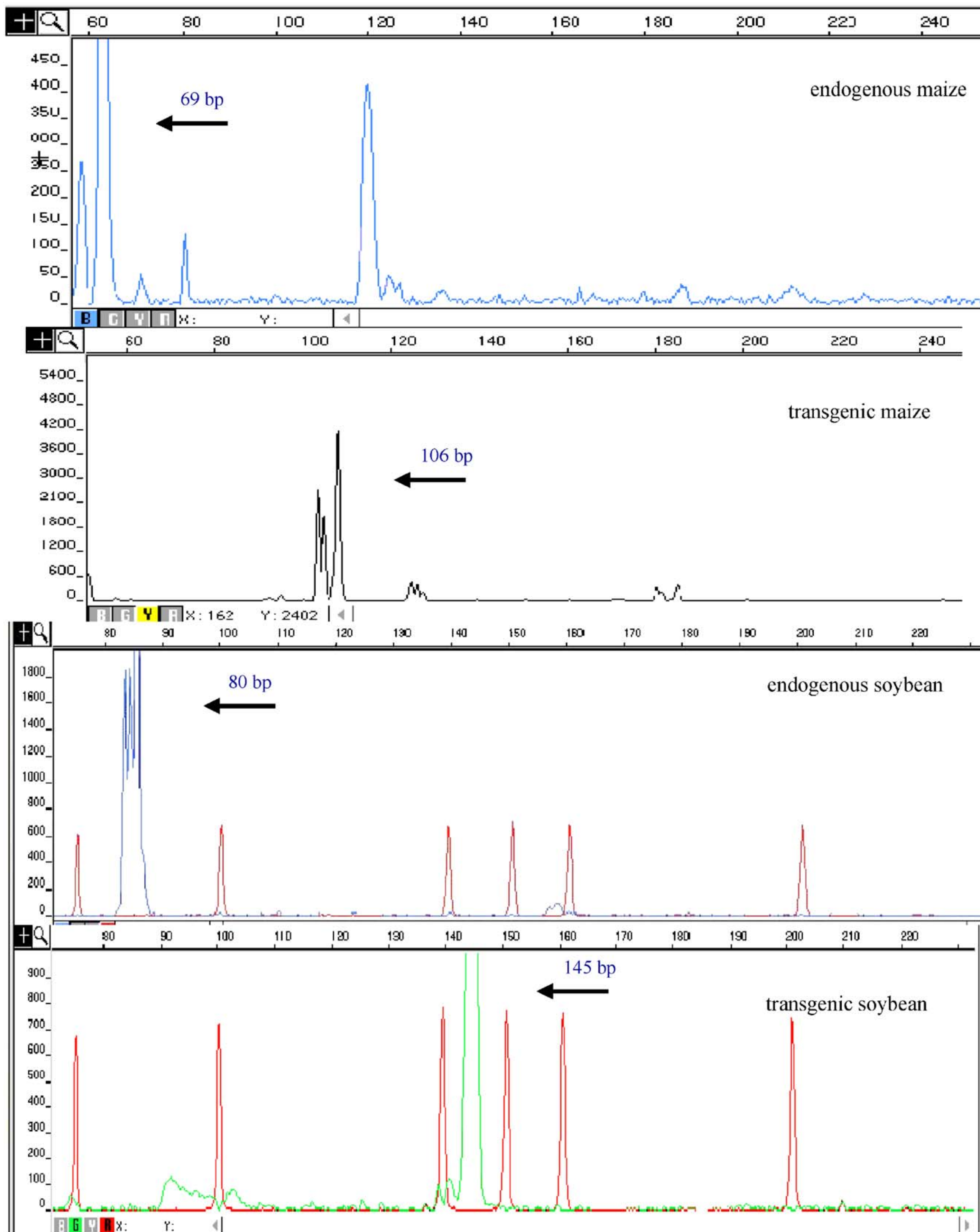

Fig. 2. Multicomponent analysis after PCR amplification with endogenous and transgenic primers. The primers labeled with the fluorescent reporter dye on the 5′ end are described in the ,,Materials and Methods'' section. Molecular weights are shown in bp (horizontal). Normalized fluorescence intensity is shown in the vertical line.

Table 1. Detection of transgenic DNA in milk samples by PCR followed by gel electrophoresis or multicomponent analysis (A to N, different milk brands tested)

Milk samples	Type of milk	No. of sample analyzed	*Cry1A*		*CP4EPSPS*	
			GE	MA	GE	MA
A_1	Normal	5	3	1	3	3
A_2	Normal	3	2	1	0	0
A_3	Organic	4	2	1	2	2
B_1	Normal	3	1	0	1	1
B_2	For children	2	0	0	0	0
C_1	Normal	3	1	0	1	1
C_2	Normal	1	0	0	0	0
C_3	Organic	4	1	1	0	0
D_1	Normal	5	3	2	0	0
D_2	Normal	3	2	2	0	0
D_3	Normal	1	0	0	0	0
E	Normal	2	1	1	0	0
F	Normal	2	0	0	0	0
G_1	For children	2	0	0	0	0
G_2	For children	2	0	0	0	0
H	For children	3	1	1	0	0
I	Organic for children	3	1	1	0	0
L	Organic	3	2	1	0	0
M_1	Organic	3	1	1	0	0
M_2	Organic for children	3	1	1	0	0
N	For children	2	1	1	0	0
P	Sheep	1	0	0	0	0
Total		60	23	15	7	7
%			38.3	25.0	11.7	11.7

Table 2. Detection of endogenous DNA in milk samples by PCR followed by gel electrophoresis (A to D, different milk brands tested

Milk samples	Type of milk	Maize		Soybean	
		Zein	*Cry1A*	*Le 1 lectin*	*CP4EPSPS*
A_2	Normal	+	+	+	–
A_3	Organic	+	+	+	+
B_1	Normal	–	+	–	+
C_1	Normal	–	+	–	+
D_1	Normal	–	+	+	–
Total		2	5	3	3
%		40.0	100.0	60.0	60.0

(60.0%). The concordance of endogenous and transgenic sequences detection, that is the proportion of positive/positive and of negative/negative was of 60.0% for maize and 20.0% for soybeans (Table 2).

Discussion

As far as the authors are aware, the present study is the first to report the presence of GM DNA in milk samples collected from the Italian market. The method used in our survey shows a very high sensitivity and the use of an automatic identification of the amplified products increases the specificity and reliability of our screening methodology. Furthermore, although occurrence of false positive signals has been demonstrated by Klaften et al. (2004), control tests were always included alongside our assays, thus excluding laboratory contamination during sample preparation or analysis. A number of studies have now been conducted in which GM DNA has not been detected in milk, meat, or eggs

derived from livestock receiving GM feed ingredients (Phipps and Beever, 2001; Phipps et al., 2002, 2003). A possible explanation may be that the diet-derived DNA is degraded in the digestive tract so that only very small fragment sizes were present, and/or to the low sensitivity of the employed extraction methods and PCR assays.

The rationale for choosing to amplify very short fragments (106 bp for GM maize; and 145 bp for GM soybean) was that ingested DNA sequences would be degraded, although not completely, in the gastrointestinal (GI) tract. Recent studies suggested that the GI tract of mammals is not a complete barrier to the uptake of small fragments into the bloodstream (Poms et al., 2003; Schubbert et al., 1997) and Chowdhury et al. (2004) suggested that although feed-derived maize DNA was mostly degraded in the GI tract still fragmented DNA was detectable in the GI contents; as such it may serve as a possible source of transfer to milk also via fecal contamination. A recent experimental model demonstrated that GM DNA sequences from feeds are detectable in milk after aerosol contamination with feed particles (Poms et al., 2003). The probability of feed particle contamination under real conditions is affected by the milking technologies employed. In Italy, modern milking plants use self-contained systems in which small particles can find entry only through insufficient air filters. Moreover, we demonstrated that the pasteurization process is not able to degrade the DNA sequences in spiked milk samples. As such, the detection of GM sequences in milk may be interpreted either as a biomarker of transgene feeding via fecal contamination with GM DNA, or, if we exclude it accounts for the ingestion of GM feed, it may indicate the presence of GM feed in the vicinity of milking and milk-storage facilities. In the European Union, the consumer is offered the option to choose between traditional foods and such that contain or consist of GM organisms by legally regulating the labelling of foods and feeds. In addition, the use of GM organisms in organically grown products is prohibited (European Commission, 1999). As such the presence of GM sequences in organic milk may indicate poor-quality assurance by the farmers and/or the producers.

One more issue, at least for the *Cry* sequences, is that one theoretical source of alternative contamination could be the environment: *B. thuringiensis*, the soil microorganism from which the sequence has its natural origin, could be an environmental contaminant of milk. Likewise, *Agrobacterium* sp. from witch the *CP4PSPS* sequence of GM soybean has been isolated could, at least theoretically, contaminate the environment around milking and milk-storage facilities. The low level of concordance between the presence of endogenous and transgenic maize and soybean genes highlighted by our results, even on a limited number of milk samples, may support an alternative source of contamination, possibly recognizable in soil bacteria of the natural environment.

Further studies performed on a larger number of milk samples are needed to understand the likely source of contamination of milk collected from the Italian market.

In this context, it should be noted that the safety of DNA and protein introduced into genetically enhanced agricultural products is based on strong scientific principles and premarket regulatory assessments (FAO/WHO, 1991, 1996; OECD, 1998, 2000) and that the Food and Agriculture Organisation (FAO) and the World Health Organisation (World Health Organisation (WHO), 1993) of the United Nations had concluded that there is no inherent risk in consuming DNA, including that derived from GM crops. This view is based on the long history of safe consumption of significant quantities of DNA from a wide variety of sources including plants, animals and microbes.

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