Detection of Genetically modified DNA in Milk Using Species Specific PCR

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Abstract: Milk has many nutrients that make it an ideal food all over the world and Iraq is one of the developing countries who imports milk to supply the people with this important food.And because most milk producer in the world depend on genetically modified (GM) feed to fed their animals, so these GM DNA may transfer to the products of these animals including milk. To detect of the purity of milk from GM DNA fragments this study was carried out,by collecting seven Samples of imported milk powder from the Sulaimani market in Iraq, which include: Anchor, Mudhish, Maraey Al-khadra, Dielac (Ireland), |Nido, Premier and Dielac (Neusland) and a fresh milk sample from cow, sheep and goat from private farm k .Suitable amount of DNA was obtained using by CTAB method from all the samples, To evaluate the feasibility of the PCR method for detection of GM DNA in milk, Species specific primer targeting the most common GM gene associated with feed including NOS-terminator of *bar* inserted gene in herbicide tolerant Event 176 GM-maize and the 35S-promoter of EPSPS inserted gene in pest resistance Roundup Ready GM-soybeans. The presence of the transgenic sequences of maize of the expected size (184bp) was reproducibly amplified in three samples whereas no amplified band was obtained using the other set of primers which are specific to the GM-soybeans. The data revealed that the PCR method can sufficiently detect GM DNA in milk powder to differentiate the GM Products from non-GM one.
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1. Introduction

In order to fulfill the requirements of the legislation on labeling, reliable and sensitive methods for detecting GM DNA in food and feed ingredients are needed, there are many DNA based technique that used, among them PCR (Polymerase chain reaction) is preferred for its sensitivity and specificity . Since the commercial production of Genetically Modified (GM) foods at the end of 1990s, which some of them are considered potentially risky for human health, consumers, especially in Europe, have shown increasing concern over such food, This makes the producer use a lot of them as feed, and since the absorption of plant DNA including GM DNA across the intestinal barrier is a natural event, as demonstrated by the detection of endogenous plant genes in several animal tissues (Tudisco *et al.,* 2006), and the product of the animals which eat this kind of food including milk (Agodi *et al.,* 2006).

As GMF has been commercialized worldwide, there should be no concerns about them before this time, thought that their material is degraded during processing into feed and during digestion (Phipps *et al*., 2002).Nevertheless, several of these studies found that plant chloroplast DNA from animal feed is present in milk, eggs and meat (Chowdhury *et al*., 2004), also Plant DNA from feed has been detected in muscle of chickens fed GM feed (Einspanier *et al*., 2001)' and organs of calves Fed GM feed (Chowdhury *et al*., 2004), so the European Union regulation presumbly also preserve the consumer's choice to avoid GM ingredient in food in addition to the animal that may have eaten it, and because a lot of GM Feed produced recently that may be used in diets for productive livestock,the possible transfer and accumulation of novel DNA in food for human consumption derived from animals receiving GM feed recently was the object of scientific dispute (Beever and Phipps, 2001; Kotowicz *et al*., 2008).

Any investigation into whether modified DNA or novel proteins consumed by animals has the potential to affect animal health, or to enter the food chain, should consider the fate of these molecules within the animal (Agodi *et al*., 2006). A Canadian team fed pigs and sheep Roundup Ready oilseed rape and then examined various tissues from the animals; they found that a liver, a kidney and intestinal tissues from the pigs, and intestinal tissues from the sheep contained fractions of the transgenes (Sharma *et al*., 2006). In another study, Italian scientists fed piglets for 35 days on Monsanto's GM maize; they subsequently found fragments of a transgene in the blood, liver, spleen and kidney of the animals (Mazza *et al*., 2005). Also Einspanier, (2001) detected GM material (from GM soya and GM maize) in the milk of cows which had been fed large amounts of GM plants, Recently fragments of the GM soybean (Roundup Ready) were detected in several blood and milk samples of goats and fragments of both of the 35S promoter and the CP4 EPSPS gene were

detected in liver, kidney and blood. (Tudisco *et al*., 2010).

Moreover, due to the great stability of genomic DNA, sterilization or pasteurization could not be effective for the full degradation of the molecule, therefore, could maintain full functional integrity as Italian scientists demonstrated the presence of GM maize sequences in milk samples collected from the Italian market (25%) and of GM soybean sequences in milk (11.7%) (Agodi *et al*., 2006). Because little information is available at the DNA level on the quality of milk powders imported to Kurdistan region of Iraq, this study conducted to evaluate the species specific PCR to investigate the quality of the different brands of milk powder in order to grade their quality by detect their purity from genetically modified DNA especially from GM maize and GM soya.

2. Materials and Methods

2.1. DNA extraction from milk

Seven Samples of milk powder were collected from the Sulaimani market including :Anchor, Mudhish, Maraey Al-khadra, Dielac (Ireland), Nido, Premier, Dielac (New Zealand), and three fresh milk samples of Sheep, Goatand Cow milk. DNA from milk was extracted by CTAB method as described by He *et al* (2007) with slight modification: 1ml of the milk sample was mixed with an equal volume of CTAB extraction buffer [1.4 M NaCl, 2% CTAB (cetyl tri methylammonium bromide), 100 mMTris, 20 mM EDTA, pH 8.0], 2% of 2-mercaptoethanol and 100 µg/ml of Protenase K for 1 h at 65ºC with shaking. Then the samples were extracted with 2 ml of chloroform, centrifuged for 30 min at 4000rpm.the supernatant precipitated with 2 volumes of CTAB precipitation buffer (40 mM NaCl, 0.5% CTAB) and incubated at room temperature for 60 min, and centrifuged for 30 min at 4000rpm.the pellet was dissolved in 300 μL of 1.2 M NaCl and extracted with an equal volume of chloroform, then the mix centrifuged for 30 min at 4000rpm.DNA in the aqueous phase was precipitated with 0.1 volume of Ammonium acetate and 2 volume of isopropanol .then the precipitated DNA was transferred into a fresh tube adding 500 µl of (70%) ethanol, and washed. Finally, it was dissolved in TE (Tris, EDTA) buffer.

2.2. PCR Reaction

The sequences of specific PCR primers for GM Soya which designed on previously published paper (yoke-kqueen et al, 2011) were as follows: (forward; 5'-TGGCGCCCAAAGCTTGCATGGC-3') and (Reverse; 5'-CCCCAAGTTCCTAAATCTTCAAG-3') Which expected to produced 356 bp fragment and the sequences of the specific primer for GM Mize were: Forward; 5'-ACCATCAACAGCCGCTACAACGA-3' and Reverse; 5'-TGGGGAACAGGCTCACGATGTCC-3'. All primers were purchased from Bionerr Company (Korea). PCR reactions were performed with 1×PCR buffer without MgCl2; 2.5 mM MgCl2;50ng DNA, 0.3 µM each primer; 0.25 mM (each) dNTP; 25 U/ml Taq DNA polymerase (sigma). PCR reactions were carried out in PCR thermal cycler (Biotech, U.K). Amplification consisted of 35 cycles: 1 min at 94°C, 1 min, 1 min at 60°C, and 1 min at 72°C. The amplified products were electrophoresed in a 1.2% agarose gel and were subsequently visualized by UV illumination after ethidium bromide staining (sambrok *et al*, 2000).

3. Results and Discussion

3.1. DNA Extraction from milk

The full amount of DNA obtained in this study (Figure 1) refers to the successful method of DNA isolation from fresh and powder milk, which depend on using the protocol of Nemeth *et al.* (2004). The efficiency of this method to obtain suitable amount of DNA is due mainly to the highly versatile cetyl trimethylammonium bromide (CTAB), because it is a detergent that helps lyses the cell membrane and make a complex with the DNA in the sample to protect it from the next steps of DNA extraction.

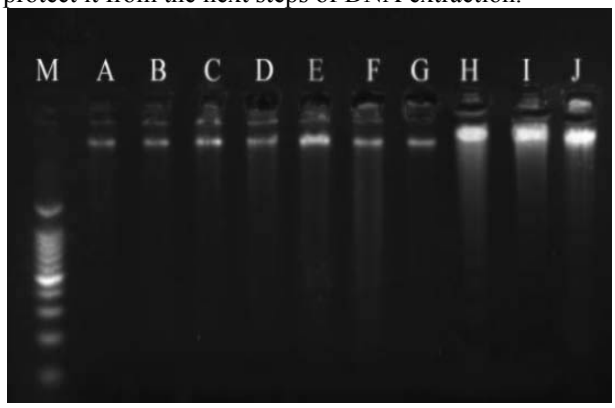

Figure 1: Represent the results of DNA extraction from ten samples of milk, that was performed on (1.2 %) Agarose gel electrophoresis and run at 90 volt/cm for one hour.Lane (M) = 1kb DNA ladder, lane (A) Anchor (B) Mudhish (C) Maraey Al-Khadra (D) Dielac Ireland (E) NIDO (F) premier (G) Dielac New Zealand (H) Sheep milk (I) Goat milk (J) Cow milk.

3.2 PCR analysis

Although many varieties of GM-soybeans and GM-maize produced for commerce, however, only two varieties, Roundup Ready GM-soybeans and Event 176 GM-maize, are now the major products on the market (Lin *et al*., 2000). PCR and

Gel electrophoresis analysis, the presence of the transgenic sequences of maize of the expected size (184bp) was reproducibly amplified in three samples (Anchor, Maraey Al-Khadra and Dielac (New Zealand) from the ten samples used in this study (Figure 2), using the primers, NOS-terminator of *bar* inserted gene in herbicide tolerant Event 176 GM-maize, whereas no amplified band was obtained using the other set of primers which are specific to the 35S-promoter of EPSPS inserted gene of glyphosate tolerant Roundup Ready GM-soybeans which were selected for PCR analysis . PCR is a highly reproducible and sensitive technique that can be successfully used in detecting transgenic for screening GM soybeans and GM maize (Randhawa and Firke 2006).The main propose of depending on DNA based techniques to detect GM DNA rather than protein is that nucleic acids are very thermo-stable molecules whereas proteins are thermo sensitive molecules. Upon processing, food proteins are no longer detectable or are detectable with difficulty because they are degraded. Conversely, nucleic acids are only slightly damaged by heat treatment. According to Meyer *et al*. (1999), by comparison, the ELISA-test which is based on using antibodies against specific proteins may be around 100 times less sensitive than the PCR method, with the advent of PCR, a specific nucleic acid can be detected whatever the foodstuff analyzed, even in mixtures where the GMO ingredient is present in low concentrations.

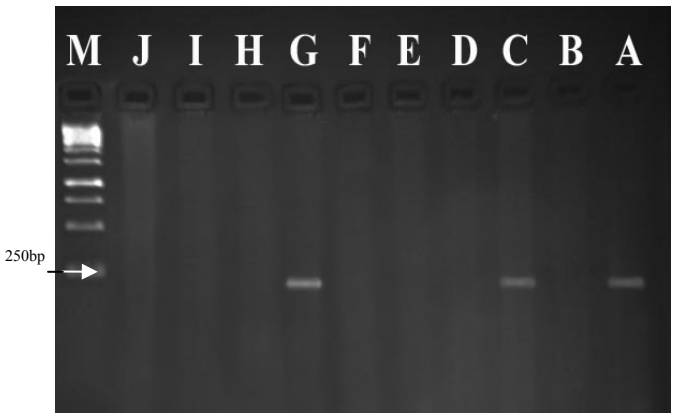

Figure 6: Represents amplified product with 184 bp using maize specific primer in 10 sample of milk. Lane (M) = 1kb DNA ladder, lane (A) Anchor (B) Mudhish (C) Maraey Al-Khadra (D) Dielac Ireland (E) NIDO (F) premier (G) Dielac New Zealand (H) Sheep milk (I) Goat milk (J) Cow milk, and Gel electrophoresis was performed on (1.2%) Agarose gel and run at 90 volt/cm for one hour.

In principle, all feed contains considerable amounts of DNA and foreign DNA is not different from other sources of DNA When DNA is released from plant material by normal digestion processes that take place in the gastrointestinal tract, the ingested DNA is rapidly cleaved into small fragments by the mechanical processes of gastrointestinal enzymatic digestions and acid hydrolysis. DNA is digested into fragments and nucleotides, and it is clear that the uptake of DNA fragments from the intestinal tract into the body is a normal physiological process for animals (Lutz *et al*., 2005). The rationale for amplifying the short fragments (184 bp) of GM maize was that ingested DNA sequences would be degraded, although not completely, in the gastrointestinal (GI) tract, so it uptake's small fragments into the bloodstream (Poms *et al*., 2003).

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. it is also reported, that DNA fragments are able to cross the intestinal barrier into the blood stream (Alexander *et al*., 2007). On the other side, a rapid degradation throughout the gastrointestinal tract takes place (Wiedemann *et al*., 2006), which might explain the absence of novel DNA in blood after feed intake as there is no amplified product obtained using the primes that target the genetically modified soy bean which have a large molecular size (356 bp).

So species specific primers targeting small fragment in milk recommended, this is in line with (Agodi *et al.,* 2006). The low number of copies of GM DNA would also hinder to trace a possible transfer of recombinant DNA into the blood stream (Bertheau *et al*., 2009).

A number of studies have now shown that DNA (including GM DNA) can survive digestion and be found in the tissues of animals eating it (Schubbert *et al*., 1997; Einspanier *et al*., 2001; Mazza *et al*., 2005). Moreover, a recent survey of milk on Italian supermarket shelves found GM DNA in over a third of milk samples tested (Agodi *et al.,* 2006). This means that these researchers were able to determine which GM crops the cows had eaten by looking into their milk.

Some studies detect Chloroplast DNA in the blood of animal instead of GM DNA, this is due that the DNA contained in the nuclei of cells which is where the novel genes (‘trangenes’) are usually inserted for making GM crops. It was instead the DNA that is found in the chloroplasts, which are present in large numbers in plant cells, and it is vastly more abundant than nuclear DNA, since each plant cell can have thousands of copies of chloroplast

genes but just two to four copies of each nuclear gene.Plant chloroplast DNA is therefore thought to be more detectable in animal products than nuclear DNA simply because of its greater abundance, not because it is less susceptible to breakdown during processing or digestion.It is therefore in fact likely that many studies were failing to detect GM crop ('transgenic') DNA in animal products and tissues because of its comparatively low level of presence and limitations in the sensitivity of the analytic methods being used, rather than transgenic DNA does not actually make its way into animal products and tissues. So using more efficient for detect GM DNA recommended such a real time PCR. Other studies also determined the presence of GM DNA in different parts of the ruminant digestive tract (Faust, 2000). On the other hand other studies have found that GM DNA has not been detected in milk, meat, or eggs derived from livestock receiving GM feed ingredients (Phipps *et al*, 2002). A possible explanation for those 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 used in those studies. So there is ample evidence in the peer-reviewed scientific literature that meat and milk (and hence cream and cheese) from animals fed GM feed contain GM DNA from the feed, therefore, according to the current Food Standards Code, all products from animals fed GM feed should be labeled, including meat, milk, cheese and eggs, even honey from bees that have foraged on GM crops has also been shown to contain GM DNA and therefore also needs to be labeled .so this study conclude that GMDNA from the feed transfer to the animal products which can be detected by PCR.

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