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Detection of Sesame Allergen Traces with Two PCR Assays - The Challenge to Protect Food-Allergic Consumers

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A B S T R A C T

The purpose of this study was to investigate the possible presence of sesame in commercial foods normally carrying no warning for the allergen, but which may have been subjected to contamination during processing. One hundred units of widely consumed goods with high potential to contain allergenic substances deriving from nuts were analyzed, using sensitive and capable PCR (C-PCR) and Real Time PCR (RT-PCR) methodologies. Of the products examined, 15 (15.0%) declared the presence of sesame, 36 (36.0%) carried no food allergy label, 44 (44.0%) were marked by the phrase "may contain traces of nuts" and 5 (5.0%) carried the indication "may contain sesame traces". The sesame-positive products detected using the C-PCR method were 15 (100%), 12 (33.3%), 14 (31.8%) and 3 (60%), respectively. Using the RT-PCR technique, positive results were obtained for 15 (100%), 18 (50.0%), 18 (20.5%) and 5 (100%) samples, respectively. The results indicate that the PCR methods applied are highly sensitive and selective, which makes them suitable for the detection of sesame traces in food samples. In addition, they can be useful for monitoring the effectiveness of cleaning processes in the production units of the food industry.

Introduction

Food allergens pose an important health problem to our society; 6-8% of young children and about 3% of adults suffer from allergic reactions to food. Small amounts of allergens are known to induce an allergic response which can be as severe as life threatening anaphylaxis (Sampson, 2004; Crespo et al., 2006). Correct food labelling is the only way to help sensitized persons to avoid consumption of the allergenic food. Sesame (*Sesamum indicum*) is one of the 14 potentially allergenic foods which have to be declared on food labels according to the EU directive 2000/13/EC amended by 2003/89/EC. Sesame is known as a functional food ingredient because of its high content in phytochemicals (Kanu et al., 2007). However, sesame seeds are known to contain several allergenic proteins (Cohen et al., 2007; Perkins, 2001). Ses i 1 and Ses i 2 belong to the group of the 2S albumins (Pastorello et al., 2001). They have a molecular weight of 9 and 7 kDa respectively. Ses i 3 is a 7-vicilin-type globulin which belongs to the 2S albumins and has a molecular weight of 45 kDa (Beyer et al., 2002). Ses i 4 and Ses i 5 are oleosins occurring in sesame oil and posing a risk for sesame allergic persons. Leduc et al. (Leduc et al., 2006) identified two allergens with molecular weights of 17 kDa (Ses i 4) and 15 kDa (Ses i 5). In 2007, Beyer et al. (Beyer et al., 2007) identified another two allergens of sesame, Ses i 6 and Ses i 7.Wolff et al. (Wolff et al., 2003) reported that most of their tested sera reacted to a 14 kDa protein and suggested that this protein was a major allergen of sesame. The identified protein could be associated with the 7 kDa 2S albumin which was identified by Beyer et al. (Beyer et al., 2002) because of the identical amino acid sequence of the 7 kDa protein to large subunits of the 14 kDa protein. Although the risk of allergic reaction to certain proteins normally requires their presence in foods in significant quantities, there are foods in which the adverse reaction could be

caused by very small concentrations of the allergenic protein (Koppelman et al., 1999). Particular attention should be paid during the manufacturing operations of industrially processed foods, by implementing sound hygiene practices and ensuring food separation, in order to avoid cross contamination by foodborne allergens from other products (Poms et al., 2004). Often, the potential threat arising from cross-contamination is declared on the product label by the phrase "it may contain", which is a very useful piece of information for the consumers. So far, the only choice for allergic individuals, who have already experienced adverse reactions, is to avoid the consumption of offending foods, as actual treatment is not yet available. In this case, prevention is of extreme importance. Methodologies for the detection of various food allergens already exist, which are based on diverse technologies and can be designed for different purposes. Several papers report on the development of ELISAs to detect traces of sesame in food. However, detection of allergenic or marker proteins is not necessarily the only way to demonstrate the presence of the allergic compound; in fact, the detection of another type of marker molecule, like DNA, can be an alternative method (Pedersen et al., 2008; Schoringhumer and Cichna-Markl, 2007; Holzhauser et al., 2000; Holzhauser and Vieths, 1999 Yeung and Collins, 1996). There have been only a few comparative studies focusing on the detection of allergens in food products, using different methodologies. In 2008, Mustorp et al. (2008) developed a sesame specific PCR method for the gene encoding 2S albumin - a major allergen of sesame. No cross reactivity was shown with a variety of plant materials. The limit of detection was 0.5 pg sesame DNA, with an amplification efficiency of 96%. The method allowed the detection of 0.005% (w/w) sesame in barbecue spice and wheat flour. Also, Schoringumer et al. (2009) described the development and validation of a duplex real-time PCR method allowing the simultaneous detection of traces of potentially allergenic sesame and hazelnut in different food consumables. The main objective of the present study was to implement sensitive and capable PCR (C-PCR) and Real Time PCR (RT-PCR) methodologies for the detection and quantification of sesame traces in various kinds of consumer goods. Demonstrating the efficiency and reliability of the methods to detect food allergens, would enable processors and merchandisers to formulate more effective strategies for protecting the health of allergic consumers.

Materials and Methods

Food collection

During the study period (September 2013 to June 2014) a total of one hundred units of widely consumed goods, potentially responsible for food allergies, were collected from local super markets and studied. Among them, there were samples containing sesame as an ingredient (category I), samples declaring the possible presence of sesame "traces" (category II), samples that might contain "traces" of nuts, without further information (category III), as well as samples that did not feature any food allergy label or warning on potential allergenic substances (category IV). The samples analyzed included various kinds of cereals, chocolates, biscuits, wafers and other snack foods. Natural products, such as sesame seeds, were used as positive controls, while hazelnuts and peanuts were selected as negative samples.

Genomic DNA extraction and quantification

The NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Germany) and the Bioo Scientific kit (Austin, USA) were tested for the extraction of hazelnut, peanuts and sesame seeds and commercial foods, in order to compare the extraction yield and cleanup of the genomic DNA. All extraction methods were applied according to the manufacturer's instructions with some modifications. The NucleoSpin Food kit was finally selected for the extraction of all samples. About 100 mg of each sample were used for the extraction, after grinding in liquid nitrogen. DNA concentration was determined spectrophotometrically. All samples were tested neat and diluted 10^{-1} in dH_2O.

PCR assay

The first PCR protocol was a previously reported (Koppel et al., 2010) conventional assay (C-PCR) amplifying a 133-bp fragment of the gene coding the Ses i 1 protein (Figure 1). Amplification was performed in a 50-μL volume reaction containing 25 μL of Master Mix (10x) PROMEGA (HotStart DNA polymerase) 0.4 μM of each primer sesF CCC GCT CTT CGT CAT CTT CAG, sesR GTA GTT CAG CAA CCA CGA AAT CG, 15 μL of eluted DNA, and dH_2O to make up to 50 μL. Amplification conditions consisted of an initial 5 min denaturation step at 95°C, followed by 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 54°C, and 1 min extension at 72°C, and finally a 10 min extension step at 72°C. PCR products were separated in a 3% agarose gel, stained with ethidium bromide (0.5 μg/mL), and documented under UV illumination. The second protocol was also an in-house established real-time PCR assay (16) using the primers sesF 5'-TGAGGAACGTGGACGAGAG, sesR CCCTAGCCCTCTGGTAAACC and TaqManProbe: FAM- ACCCTCCTGCTGCTGCTGCC-TAMRA; RT targeting the gene coding for the Ses i 1 protein amplifying a 117 bp fragment. Reactions were performed in a 25-μL final volume, containing 12.5 μL of Master Mix (KAPA Probe Fast qPCR, KAPA BIOSYSTEMS), 0.2 μM of each primer, 0.1 μM of TaqMan Probe and 10 μL of eluted DNA to make up to 25 μL. Amplification conditions consisted of a 10 min initial denaturation step at 95°C, followed by 40 cycles of 25 s denaturation at 95°C, 50 s annealing and elongation at 54°C.

Plotting of standard curves for real-time PCR analysis

The Step One plus™ Real Time PCR System (Applied Biosystems) was used for the PCR assay. RT fluorescence measurements were compiled in every cycle. All reactions included negative controls containing the amplification master mix and dH_2O that was used for reagent preparation.

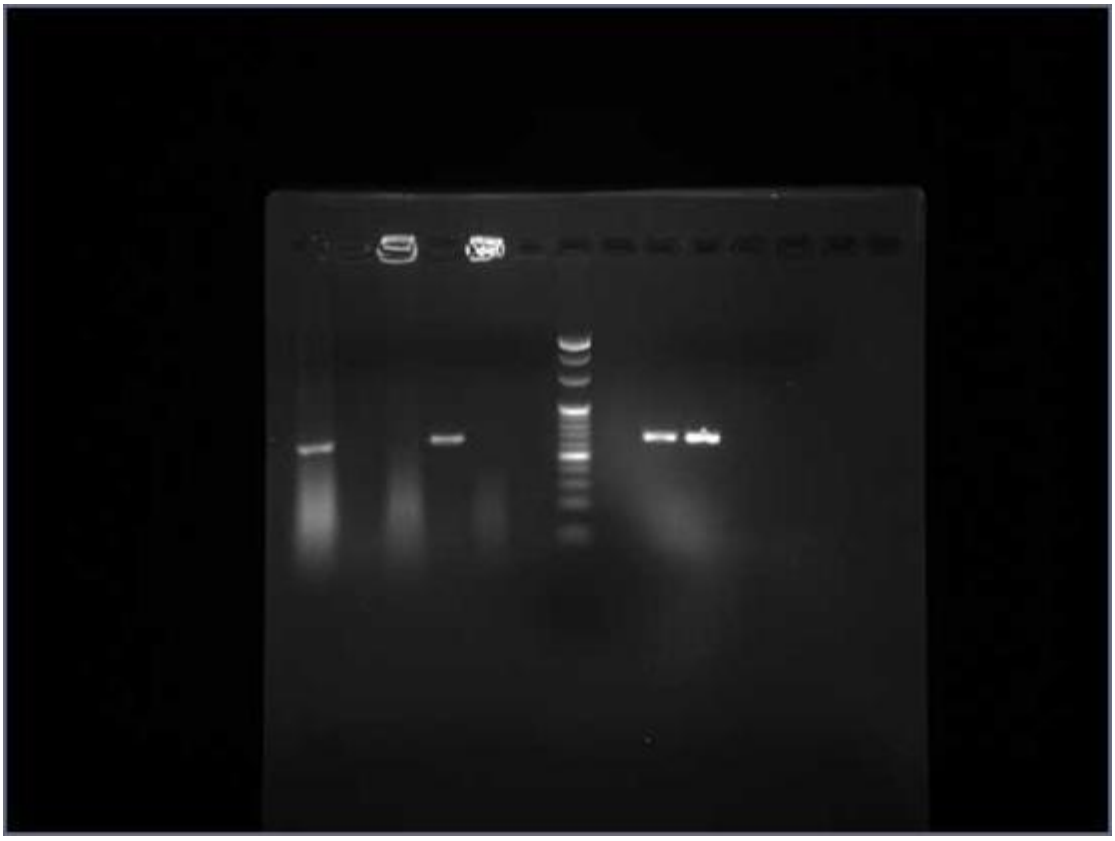

Figure 1 The arrow indicates the expected PCR product (133 bp).
Lane 1: PCR product from Wafer which contains sesame as "traces". **Lane 2**: Biscuit which contains no food allergy labels. **Lanes 3 and 4**: Snack which contains sesame as "traces" neat and diluted 10^{-1} in dH_2O respectively. **Lanes 5, 6 and 8**: Biscuit, Cereals and Wafer which contain potential allergenic substances referred to as "traces". **Lane 7**: 20 bp ladder size standard. **Lanes 9 and 10**: PCR products from sesame seeds as positive control. **Lanes 11 and 12**: Negative controls from hazelnut and peanut seeds. **Lanes 13 and 14**: Negative controls **(**dH_2O).

For positive controls and DNA quantification, a standard curve was designed using known concentrations (KC) of DNA extracted from sesame seeds (KC_1=10 ng/100 mg of food, KC_2=1.0 ng/100 mg of food, KC_3=0.1 ng/100 mg of food, KC_4=10 pg/100 mg of food, KC_5=1.0 pg/100 mg of food and KC_6=100 fg/100 mg of food). A cycle threshold value (Ct) was defined as the cycle of the RT-PCR at which a significant fluorescence increase in comparison to the negative control and the blanks was detected; this increase was associated with an exponential growth of PCR product during the log-linear phase. RT-PCR runs were acceptable only when the negative control had an undetectable Ct, the KC2 and KC3 had Cts between 25 and 27, and the efficiency of the PCR was 90-100%. All samples were tested neat and diluted 10^{-1} in dH_2O for the detection of inhibition. Inhibition was defined as a positive PCR result with a diluted specimen, while a negative PCR result was obtained with the specimen tested undiluted.

Results and Discussion

DNA Extraction and Quantification

The DNA extraction methods critically affect PCR sensitivity. The optimization of these methods can improve the detection and quantification yield of sesame traces in consumed foods. In order to achieve this, two different extraction methods were tested (data not shown) and the NucleoSpin Food kit was finally selected for sesame seeds. About 100 ng of genomic DNA were obtained from 100 mg of food (sesame seeds and commercial food samples) using the NucleoSpin Food kit.

Dynamic range, analytical sensitivity and specificity

None of the C-PCR or RT-PCR protocols produced any results using DNA extracted from hazelnut or peanuts seeds, whereas a positive signal was detected by either protocol using DNA from sesame seeds. Based on the DNA quantification performed, reproducible analytical sensitivities of RT were 100 fg/100 mg of food.

Sensitivity and specificity

Overall, one hundred units of widely consumed snack foods, which were either potentially responsible for food allergies or contained no food allergy labels or warnings for potential allergenic substances referred to as "traces", were collected from super markets and studied. The snacks analyzed included 20 units of various kinds of cereals, 12 units of chocolate products, 30 units of biscuits, 15 units of wafers and 23 units of other snacks. Sesame seeds were used as positive controls. Out of the one hundred food products tested, a total of 15 (15.0%) contained sesame as an ingredient, 36 (36.0%) did not feature any food allergy labels, 44 (44.0%) were labeled with the phrase "may contain traces of nuts" and 5 (5.0%) declared the presence of "traces" of sesame. Based on the

Table 1 Coefficient of variation values for both intra- and interexperimental test results of 5 randomly chosen samples.

Sample	Interexperimental							
	Experiment 1		Experiment 2		Experiment 3		Intraexperimental	
	Mean Ct	CV %	Mean Ct	CV%	Mean Ct	CV%	Mean Ct	CV%
1	29.72 ±0.15	0.50	30.45 ±0.18	0.59	30.68 ±0.10	0.33	30.89±0.12	0.39
2	22.74 ±0.10	0.44	23.00 ±0.08	0.35	23.10±0.12	0.52	22.98±0.20	0.87
3	23.99±0.08	0.33	23.45 ±025	0.11	23.38±0.18	0.77	23.58±0.16	0.68
4	28.57±0.06	0.21	27.95 ±0.20	0.72	27.52±0.15	0.55	27.99±0.18	0.64
5	30.01±0.14	0.47	29.45 ±0.18	0.61	29.58±0.20	0.68	29.75±0.15	0.50

results obtained using the C-PCR, 15 (100%), 12 (33.3%), 14 (31.8%) and 3 (60%) specimens, respectively, were found positive. Using the RT-PCR, the respective positive samples were 15 (100%), 18 (50.0%), 18 (20.5%) and 5 (100%). When diluted specimens were examined, 5 inhibition cases were detected. All the samples containing sesame were negative when tested neat and became positive when diluted 10^{-1} in both C-PCR and RT.

Repeatability and reproducibility of assay

In order to evaluate the repeatability and reproducibility of the method, 5 samples at a concentration of 1.0 ng/μL were randomly chosen as PCR templates and amplified in triplicate in an experiment performed 3 times. The results of the TaqMan assay showed that the coefficient of variation values for both intra-experimental and inter-experimental data ranged from 0.39 to 0.87% and 0.11 to 0.77%, respectively (Table 1). These results suggest that the method presents good repeatability and reproducibility.

DNA quantification

Analytical DNA quantification of all positive specimens, using RT protocol is shown in Tables 2, 3 and 4. C-PCR and RT-PCR resulted in a high overall sensitivity (100%). RT-PCR and C-PCR identified all 15 specimens that contained sesame as an ingredient. RT-PCR technology has been extensively evaluated in food allergens. More specifically it has been used for direct detection of allergen substances in food, using technologies like SYBR Green, and hydrolysis TaqMan probes. Nevertheless, the overall sensitivity of the RT-PCR protocol reported in this study differed from the sensitivities reported for C-PCR assays. Also, increased sensitivity is not always the main advantage of RT-PCR assays over the conventional ones, but rather the rapid reporting as a result of faster turnaround times. In addition, no post-PCR processing is necessary, and both amplification and detection are performed in a single closed tube, thus minimizing the risk of carrying over or cross-contamination. Quantification is another potential advantage of RT-PCR protocols, which nevertheless needs to be further evaluated, in order to reach any definite conclusions regarding the improvement of detection of allergen traces. As a consequence, PCR results could be used as a first screening step to select samples for further confirmation by Real Time PCR. The findings from this study also agree with relevant works published from other researchers. For example, in 2007, Schoringhumer and Cichna-Markl (1999) published a real-time PCR method for the detection of the gene coding for the Ses i 1. A molecular beacon probe was used. Seventeen samples, such as Brazil nut, sunflower seed, peanuts or black sesame, did not show any cross reactivity. Amplification efficiency of 100.3% was shown up to a serial dilution of 1:10000 of sesame DNA extracts. For the determination of the limit of detection in a food matrix, crisp bread was spiked with sesame seeds in concentrations of 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01 and 0.001%. The efficiency was 86.4%. Ct values of over 35 were obtained for sesame concentrations below 0.05%. Also Brzezinski (2007) presented a real-time PCR method for the detection of sesame in food products. The amplification of the 66 bp fragment of the 2S albumin gene (Ses i 1) was detected by using a TaqMan probe. No cross reactivity was found for seeds which are commonly used in bakery products, such as pumpkin, poppy and sunflower seeds. The limit of detection was determined to be 5 pg of DNA, which corresponds to 50 mg/kg of sesame DNA in food products.

Conclusions

The risk for the presence of allergens in foods is not associated solely with the use of allergenic raw materials as food ingredients; allergenic residues can also be present due to cross-contamination from other products or the equipment used. Food industry has a responsibility to produce foods that are safe for all consumers, including food allergic people. This would also limit the overuse of the "may contain traces of" statement by the industry, which leads many allergy sufferers to disregard label warnings, setting themselves at risk. As demonstrated, the presented PCR methods are highly sensitive and selective, which makes them suitable for the detection of small amounts of sesame traces in food samples. Moreover, these methods can also be useful for monitoring the effectiveness of the cleaning processes in the production units of the food industry.

Table 2 Results of the positive samples C-PCR and the Real-Time assays of the samples that were labeled with "may contain "traces" of nuts.

No	Specimen	C-PCR	RT	
			DNA yield (ng/100 mg food)	Ct
1	Wafer 1	Positive	0.676	28
2	Wafer 2	Positive	0.676	28
3	Wafer 3	Positive	0.122	30
4	Wafer 4	Positive	2.435	26.5
5	Wafer 5	Negative	0.056	31
6	Biscuit 1	Negative	0.022	32
7	Biscuit 2	Positive	0.656	28
8	Biscuit 3	Negative	0.056	31
9	Biscuit 4	Positive	0.122	30
10	Chocolate 1	Positive	0.288	29
11	Chocolate 2	Positive	0.122	30
12	Snack 1	Positive	0.056	31
13	Snack 2	Positive	0.676	28
14	Snack 3	Positive	0.676	28
15	Snack 4	Positive	0.288	29
16	Cereal 1	Positive	0.122	30
17	Cereal 2	Positive	0.122	30
18	Cereal 3	Positive	0.676	28

Table 3 Results of the positive samples of C-PCR and the Real-Time assays of the specimens that did not feature any food allergy labels.

No	Specimen	C-PCR	RT	
			DNA yield (ng/100 mg food)	Ct
1	Wafer 6	Positive	0.122	30
2	Wafer 7	Positive	0.656	28
3	Snack 5	Positive	0.656	28
4	Snack 6	Positive	0.656	28
5	Snack 7	Negative	0.056	31
6	Snack 7	Negative	0.056	31
7	Snack 9	Positive	0.656	28
8	Snack 10	Negative	0.056	31
9	Chocolate 3	Negative	0.056	31
10	Chocolate 4	Negative	0.056	31
11	Chocolate 5	Negative	0.022	32
12	Chocolate 6	Positive	0.676	28
13	Chocolate 7	Positive	0.676	28
14	Biscuit 5	Positive	0.122	30
15	Biscuit 6	Positive	0.145	29.8
16	Cereal 4	Positive	0.145	29.8
17	Cereal 5	Positive	0.676	28
18	Cereal 6	Positive	0.676	20.8

Table 4 Results of positive samples with C-PCR and the Real-Time assays of the specimens that declared to contain "traces" of sesame.

No	Specimen	C-PCR	RT	
			DNA yield (ng/100 mg food)	Ct
1	Wafer 8	Negative	0.022	32
2	Snack 11	Positive	0.656	28
3	Snack 12	Positive	1.589	27
4	Biscuit 7	Positive	0.441	28.5
5	Biscuit 8	Negative	0.122	30

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