Evidence for Wheat, Rye, and Barley Presence in Gluten Free Foods by PCR Method – Comparison with Elisa Method

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Abstract

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A method of the evidence for the presence of wheat, rye, and barley in gluten free foods, based on the polymerase chain reaction (PCR), was validated. DNA was isolated from foods by chaotropic solid phase extraction. The PCR method applied was focused on the intron of the chloroplast gene *trnL* and utilised primers WBR11 and WBR13. Electrophoresed wheat and rye DNAs were characterised by a 201 bp fragment, barley DNA by a 196 bp fragment. The validated PCR method was applied to the selection of 18 gluten free foods, previously found by ELISA method to contain 1 mg or more of gliadin per 100 g food. The presence of wheat was confirmed by PCR method in all foods analysed. The comparison with the results obtained by ELISA method reliably verified the detection limit of PCR method, i.e., 0.02% wheat.

Keywords: polymerase chain reaction; ELISA; wheat; rye; barley; gliadin

Gluten is defined as a fraction of wheat, rye, barley, or oat proteins which is insoluble in water and in 0.5 M NaCl. The prolamin fraction is defined as a fraction of gluten extractable by 40–70% ethanol and forming 50% of gluten (Codex Alimentarius Commission 2007). The proteins of wheat gluten are constituted by reserve, water insoluble gliadins (prolamin fraction) and glutenins (glutelin fraction). The individuals suffering from celiac sprue may have serious health problems after the consumption of even a small quantity of cereals containing gluten (Wieser & Koehler 2008). This is why it is very necessary to label foods properly. According to the present legislation of the Czech Republic, gluten free foods must not contain more that 100 mg gluten per kg food ready for consumption, which roughly corresponds to 0.1% of wheat (Vyhláška č. 157/2008 Sb.).

Immunochemical methods like ELISA or lateral flow analysis are most frequently used to detect the presence, or to determine the content, of gluten. Other methods useful for determining gluten proteins are electrophoretic, chromatographic, or mass spectrometry based methods (Hulín *et al*. 2008). DNA analysis of the respective cereal by the polymerase chain reaction (PCR) method constitutes a suitable alternative for detecting the presence of toxic protein in a sample. It exceeds ELISA methods in sensitivity. Its shortcoming consists in the required presence of DNA in the sample and selective detection of its contamination with exactly one single kind of cereal. The first PCR method of qualitative determination of wheat in foods was published by Allmann *et al*. (1993). Up to now, a number of papers have been published focused on wheat and other cere-

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als detection by real-time PCR (Dahinden *et al.* 2001; Sandberg *et al.* 2003; Terzi *et al.* 2004; Piknová *et al.* 2008; Zeltner *et al.* 2009). The majority of papers comparing gliadin values in gluten free foods found by different methods have appeared only in the past ten years (Dahinden *et al.* 2001; Henterich *et al.* 2003; Sandberg *et al.* 2003; Olexová *et al.* 2006; Gélinas *et al.* 2008; Piknová *et al.* 2008).

The aim of this work was to optimise the PCR method for wheat, barley, and rye determination, and to validate it on selected samples of gluten free foods, shown by ELISA method to contain more than 1 mg gliadin per 100 g of food.

MATERIAL AND METHODS

The samples of gluten free foods for analyses were obtained from various sources. Some of these were purchased by patients reliant on the gluten free diet and were subsequently analysed as part of monitoring their dietary gliadin intake, other foods were obtained directly from the producers or purchased from retail outlets (the samples are listed in Table 1). Samples No. 17 and 18 were prepared from gluten free ingredients at home by patients on gluten free diet. Sample analyses for gliadin using ELISA and for the presence of wheat, barley, and rye using PCR method were performed in the second half of 2007 and in the first half of 2008.

ELISA method (Gliadin ELISA kit – Ref. IM3717). The format of the ELISA kit for gluten determination in food products and raw materials is a two-step sandwich assay based on two monoclonal antibodies used for solid-phase coating and signal conjugate of polyclonal antibody with horse radish peroxidase. The detection limit of 3 mg/kg for sample dilution 1:100 was determined for this kit.

DNA extraction. DNA was isolated from the food samples using either the GENESpin kit from GeneScan Co. (Freiburg, Germany), applying certain modifications by Gryson *et al.* (2004), or the NucleoSpin Food kit from Macherey-Nagel Co. (Düren, Germany). The instructions for both isolation kits utilise chaotropic solid-phase extraction which was applied as follows.

The homogenised food sample was extracted using lysation buffer and proteinase K. RNAse A was added in the course of extraction to eliminate RNA from the sample. The mixture was centrifuged to remove contaminants and cell residues. Clear supernatant was then mixed with binding

Table 1. Gliadin contents (ELISA method) and PCR reaction in selected samples of gluten free foods

Sample No.	Food item	Gliadin content* (mg/100 g) ELISA	PCR reaction
1.	maize flour	7.5	++
2.	maize flour Natural	1.2	+
3.	maize flour	4.7	+++
4.	amaranth flour Natural	6.2	+++
5.	wholemeal chickpea flour	22.2	+++
6.	wholemeal millet flour	4.7	+++
7.	buckwheat flour	52.2	+++
8.	buckwheat grits	10.2	++
9.	maize pasta	2.3	+
10.	maize and rice pasta	2.1	++
11.	bread mix Nominal	2.6	+
12.	gluten free white bread mix	1.2	+
13.	gluten free multigrain bread mix	1.4	+
14.	gluten free millet bread mix	1.1	+
15.	gluten free dumpling mix	1.1	+
16.	flax seed bar Fit Bela	2.6	++
17.	cake with lemon icing	3.8	++
18.	apple pie	1.6	+

*The coefficient of variation resulting from replicate measurement of samples did not exceed 20%

buffer and ethanol. This mixture was transferred to the mini column from the kit, which was then washed with two different washing buffers to remove potential PCR inhibitors. DNA was then eluted from the column with elution buffer or water. The isolated DNA was kept in a refrigerator at 4°C prior to subsequent amplification. The purity and concentration of the isolated DNA was assessed spectrophotometrically (BioPhotometer 6131, Eppendorf, Hamburg, Germany) by determining the absorbance ratio at 260 nm and 280 nm.

PCR. The polymerase chain reaction was performed in Touchgene Gradient cycler from Techne Co. (Cambridge, UK). The selection of primers was derived from the studies by Dahinden *et al*. (2001) and Olexová *et al*. (2006). These authors described a method for the determination of wheat, rye, and barley focused on the intron of the chloroplast gene *trnL*. They used primers WBR11 and WBR13 for the detection of the PCR products of wheat and rye sized 201 bp, and of barley sized 196 bp. Although the above-mentioned authors used the same primers for determining those cereals, they indicated to have applied different concentration, temperature, and time conditions of the PCR reaction. When optimising this PCR method in our laboratory, the following reaction conditions were found most suitable:

The reaction mixture (50 µl) contained 4 U Platinum Taq DNA Polymerase Invitrogen England, 1× concentrated PCR buffer lacking Mg (containing 20mM Tris-HCl, pH 8.4 and 50mM KCl), 2.5mM $MgCl_2$ Invitrogen, 0.2mM dNTP mix Eppendorf, 0.5µM of each primer (WBR 11 and WBR 13), solution of DNA isolate, and volume made up to 50 µl with sterile water Molecular Biology Grade, without DNAs.

Forward primer WBR 11: 5'-GGT AAC TTC CAA-ATT CAG AGA AAC-3'

Reverse primer WBR 13: 5'-TCT CTA ATT TAG-AAT TAG AAG GAA-3'

The isolates from plain wheat flour and from whole meal barley and rye flours served as standards of wheat, barley and rye DNAs in PCR reactions.

The optimised temperature and time profile of the PCR reaction was as follows:

– Initial denaturation: 95°C, 5 min;
– 45 cycles with the following settings:
  denaturation 94°C, 30 s
  annealing 53°C, 30 s
  extension 72°C, 80 s;
– Final extension 72°C, 8 min.

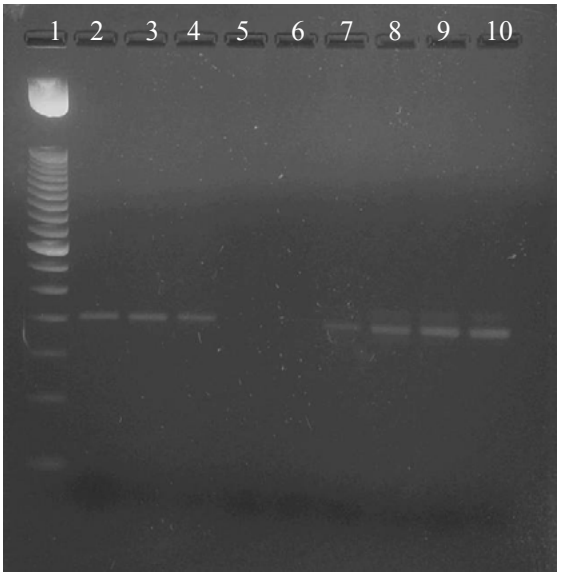
The products of the PCR reaction were analysed by gel electrophoresis on 3% agarose gel (agarose 3:1, Amresco, Solon, USA) in TBE buffer 1× concentrated and stained with ethidium bromide (0.5 µg/ml). The agarose gel horizontal electrophoresis was run at 110 V for about 90 min, along with the application of the 50 bp DNA molecular weight standard (Invitrogen, Paisley, UK), with the highlighted band at 350 bp, or possibly with low molecular DNA ladder (Biolabs) with the highlighted band at 200 bp.

Visualisation was done under UV light with a transilluminator (Herolab UVT-20, Wiesloch, Germany) and subsequently documented by a Kodak digital camera, using Kodak 1D software.

RESULTS AND DISCUSSION

The described PCR method made it possible to detect the amplified wheat DNA fragment sized 201 bp, rye DNA fragment sized 201 bp, and barley DNA fragment sized 196 bp (Figure 1).

To make sure that PCR method is applicable for detecting the presence of wheat, rye,or barley in gluten free products, it was necessary to determine its detection limit. A series of model samples of full-fat soybean flour with the admixture of plain

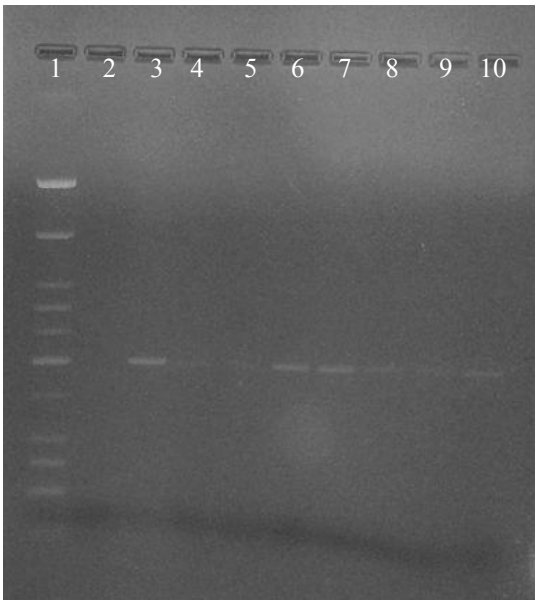


Lane 1 – 50 bp DNA Ladder Invitrogen; lane 2 – wheat DNA – 201 bp; lanes 3 and 4 – rye DNA – 201 bp; lanes 7 to 10 – barley DNA – 196 bp
Run conditions: 3% agarose gel, 1× TBE, 110 V, 1 h 35 min

Figure 1. Detection of wheat, rye and barley DNA

wheat flour were prepared. These samples were subjected to DNA isolation and amplification aimed at wheat DNA detection. Following electrophoresis, the detection limit of the PCR reaction for wheat was found to be 0.1%. This detection limit roughly corresponds to 5 mg gliadin in 100 g food and satisfies the requirements of Decree No. 157/2008 Coll. (2008). In spite of this, it was also tested whether the PCR method described could be used for the detection of wheat in foods that had been found by ELISA to contain less than 5 mg gliadin per 100 g food. For this reason, food items containing 1 mg or more of gliadin per 100 g food were selected from a large collection of gluten free foods analysed by the ELISA method. In the course of the period monitored, 18 foods and food ingredients listed in Table 1 were examined, out of which 13 items contained less than 5 mg gliadin per 100 g. These selected foods were analysed by the described optimised PCR method, which proved the presence of wheat in all 18 food samples. Electrophoresis was performed and the positive reaction for wheat was apparent even in the samples with gliadin content close to 1 mg/100 g food (Figure 2). In some cases, the electrophoretic response was less distinctive, yet this proves that the detection limit of PCR method is 0.02% of wheat (lanes 4, 5, 8, 9, 10). The values of gliadin determined by ELISA method and the results of the PCR reaction found in the selected samples of gluten free foods are presented in Table 1.

The determined 0.02% detection limit of the described PCR method is in a good correspondence with the results of other authors. Dahinden *et al.* (2001), using the same primers as the present study, determined the detection limit to be 0.1%. Olexová *et al.* (2003) found the same value and emphasised that the best isolation conditions had been achieved with the GeneSpin isolation kit. In another paper, Dahinden *et al.* (2000) detected wheat in 35 baby foods by the real-time PCR method with the detection limit ranging between 0.02% and 0.2%. Only one sample was found by both PCR and ELISA methods to contain over-limit amount of gliadin. Yamakawa *et al.* (2007) were able to detect 0.005% of wheat mixed with maize flour working with a wheat-specific gene. Debnath *et al.* (2009) described the detection of wheat by a PCR method focused on the glutenin gene with the detection limit of 0.1%. Hirao *et al.* (2009), using the ITS region primers, detected the presence of 50–500 fg of wheat DNA.



Lane 1 – low molecular DNA Ladder Biolabs (highlighted band 200 bp); lane 2 – PCR negative control; lane 3 – wheat flour standard; lane 4 and 5 – sample 2 – maize flour; lanes 6 and 7 – sample 4 – amaranth flour; lane 8 – sample 15 – gluten free dumpling mix; lanes 9 and 10 – sample 14 – gluten free millet bread mix
Run conditions: 3% agarose gel, 1× TBE, 110 V, 1 h 30 min

Figure 2. PCR analysis of gluten free foods for presence of wheat

Similar to the present study, other authors also compared the results of detecting the presence of gluten-containing cereals in gluten free foods, obtained by various methods. In 1991 and 1992, Jermini *et al.* (1994) analysed 192 gluten free foods for the presence of gliadin using ELISA and PCR methods. Their study showed that 74% of local Swiss gluten free bakery products were contaminated with wheat. Köppel *et al.* (1998) tested the suitability of PCR and ELISA methods for determining oat flour contamination with wheat on 30 samples. PCR method showed a 10-fold sensitivity compared to ELISA, ranging between 0.001% and 0.01% of wheat. Out of 9 real foods examined, Yamakawa *et al.* (2007) found wheat to be present in 8 samples by both ELISA and PCR methods. Dahinden *et al.* (2001) compared the sensitivity of PCR and ELISA for detecting wheat, barley, and rye on 15 samples. Out of these, 11 samples yielded the same results and both methods correlated well with each other. Similarly, a good correlation between both methods was found by Dahinden *et al.* (2000), when analysing 35 baby

foods. Sandberg *et al*. (2003) used real-time PCR to determine food contamination with gluten. The results of PCR correlated well with the values obtained by ELISA, but it was impossible to detect DNA in hydrolysed products (bear, syrup and malt extract). Piknová *et al*. (2008) analysed 49 gluten free foods by ELISA and by the real-time PCR focused on the gene coding for puroindoline b. They found 3 positive samples with both methods and one sample with the sole PCR method. In the course of 2005, Gélinas *et al*. (2008) monitored the Canadian market for gluten free cereal foods contaminated with gluten. They used both ELISA and real-time PCR. Out of 148 foods examined, they found about 10% of samples to contain about 200 ppm of gluten. Henterich *et al*. (2003) described a PCR method with immunological detection for gliadin determination and applied it to three food samples, comparing its results with the values obtained by ELISA. The gliadin content found was dependent on the extraction conditions of these methods. If the same way of extraction was used, the results of both the real-time iPCR and the sandwich ELISA were very similar.

CONCLUSION

A PCR method to prove the presence of wheat, rye, and barley in foods was elaborated and optimised. The wheat and rye DNAs were characterised by a 201 bp fragment, barley DNA by a 196 bp fragment. The optimised PCR method was applied to 18 selected gluten free foods, which were previously proved by ELISA to contain gliadin in the range of 1.1 mg/100 g to 52.2 mg/100 g, roughly corresponding to 0.02–1% of wheat. The presence of wheat was proved by PCR method in all 18 samples. This determined the detection limit of the validated PCR method, i.e., 0.02% of wheat. The described PCR method can be used as an alternative to ELISA method for determining wheat, rye and barley in gluten free foods.

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