Detection of gluten-containing cereals in food by 5'-nuclease real-time polymerase chain reaction

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Summary

A real-time polymerase chain reaction (PCR)-based method for the detection of coeliac disease-causing gluten-containing cereals (wheat, barley and rye) in food is described. The method consists of DNA isolation by chaotropic solid phase extraction and the subsequent real-time PCR with primers and a 5'-nuclease (TaqMan) fluorescent probe targeted to the gene encoding for puroindoline b. The method produced positive results for 31 wheat cultivars as well as barley and rye samples, and negative ones for 18 other samples. The intrinsic detection limit of the method was 0.026 ng wheat DNA, which corresponds to approx. 1.5 haploid genomes. In model flour samples, 200 $mg.kg^{-1}$ of the wheat component could be detected, which was comparable to the detection limit of gliadin-targeting enzyme-linked immunosorbent assay (ELISA). A linear calibration line was obtained for real-time PCR in the range from 200 $mg.kg^{-1}$ to 2000 $mg.kg^{-1}$. Practical applicability of the real-time PCR method was tested by the analysis of 49 food samples, out of which 3 were found positive by both real-time PCR and ELISA, and one sample was found positive by real-time PCR only. The presented real-time PCR is useful for sensitive and selective detection of coeliac disease-causing gluten-containing cereals in food products.

Keywords

wheat; barley; rye; PCR; DNA

Gluten enteropathy (coeliac disease) is a disease caused by an inappropriate immune response to dietary gluten of wheat, barley or rye. The fraction of gluten actually responsible for the disease, which makes up about 50% of gluten, is the prolamin fraction of wheat (gliadin), barley (hordein) and rye (secalin) [1]. Prolamins of other cereals are not active in coeliac disease, including avenin of oats. This fact has been demonstrated in well-designed studies and the formerly reported information on oats intolerance in coeliac patients has been attributed to contamination of commercially available oats by wheat gluten [2, 3].

Although various grains other than wheat, barley or rye do also contain gluten, this does not cause coeliac disease. In this regard, terms "gluten-containing" or "gluten-free" are ambiguous. Unfortunately, there does not seem to be an appropriate identifier for coeliac disease-causing gluten and so the above terms are widely used and have been also implemented in the legislature [4, 5].

Since no treatment is available for coeliac disease, patients suffering from it have to exclude gluten from wheat, barley or rye from their diet [1]. For these consumers, a special category of food products designated "gluten-free" is produced, which have to meet specific requirements regarding the contents of gluten. These food products should consist of ingredients which do not contain any prolamins (ingredients other than wheat or all *Triticum* species such as spelt, kamut or durum wheat, rye, barley or their crossbred varieties) with a gluten level not exceeding 20 $mg.kg^{-1}$ or should consist of "gluten-free" ingredients from wheat, rye, barley, oats, spelt or their crossbred varieties with a gluten level not exceeding 200 $mg.kg^{-1}$ or they may be mixtures of the above, with a gluten level not exceeding 200 $mg.kg^{-1}$ [4]. An important information for coeliac patients is also provided by labelling of food with regard to contents of gluten-containing cereals, as stated in the European legislature [5].

Labelling of "gluten-free" food products should be checked and gluten contents in naturally gluten-free food products can be determined using appropriate analytical methods. For this purpose, mainly

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sandwich enzyme-linked immunosorbent assays (ELISA) are used. These are available in a kit format from various commercial suppliers [6, 7].

Because several ELISA methods suffer from certain problems such as insufficient accuracy at a low gluten level (20–200 mg.kg^{-1}) or at the analysis of heat-treated food products [6, 7], besides their improvement, alternative methods have been searched for and methods for the detection of gluten-containing cereals based upon polymerase chain reaction (PCR) have been developed [8–11]. PCR-based methods are targeted to a different analyte, i.e. do not determine gluten but rather DNA of the plant (wheat, barley or rye). In principle, PCR-based methods should be more specific than immunochemical methods, but they lack a quantitative potential because the ratio of DNA to gluten varies in grains of various species and cultivars. However, this variability is not wider than one order of magnitude [12] and so there is a potential for PCR-based methods to be useful as alternative or additional qualitative methods for the analysis of food products.

In recent years, real-time PCR with 5'-nuclease (TaqMan) probes has become preferentially used in food analysis due to its high specificity, sensitivity and closed-tube format which helps to avoid laboratory contamination. Some 5'-nuclease real-time PCR methods specific for wheat or barley were developed, although not for the purpose of the detection of gluten-containing cereals but rather for the detection of "common wheat" (*Triticum aestivum*) adulteration in durum wheat pasta [13] or as an endogenous internal control for the detection of genetically modified wheat and barley [14] or "common wheat" [15].

In this work, we describe a 5'-nuclease real-time PCR-based method for the detection of coeliac disease-causing gluten-containing cereals. The method targets the gene encoding for puroindoline b, which was used by Alary et al. for the detection of "common wheat" [13], for which we have designed a new reverse primer.

MATERIALS AND METHODS

Plant materials

Grains of wheat and barley cultivars were obtained from Dr. M. Švec, Department of Genetics, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia. Reference flours from wheat, barley and rye were obtained from Dr. F. Schwägele, Bundesanstalt für Fleischforschung, Kulmbach, Germany. Other plant materials were obtained from shops selling "gluten-free" food products in Bratislava, Slovakia.

Model samples and food products

Model samples containing 50 mg.kg^{-1}, 100 mg.kg^{-1}, 200 mg.kg^{-1}, 500 mg.kg^{-1}, 1000 mg.kg^{-1} and 2000 mg.kg^{-1} of fine wheat flour (from the market in Slovakia) were prepared from "gluten-free" flour Promix-T (Novalim, Bratislava, Slovakia). "Gluten-free" and naturally gluten-free food products were obtained from grocery shops in Bratislava, Slovakia.

DNA extraction, quantification and amplifiability determination

Food samples were homogenized using mortar and pestle, and DNA was isolated by chaotropic solid-phase extraction using the NucleoSpin kit (Macherey-Nagel, Düren, Germany) without any modification to the protocol of the kit. DNA was quantified fluorimetrically with a DNA intercalation dye PicoGreen using a Quant-iT PicoGreen kit (Invitrogen Molecular Probes, Eugene, Oregon, USA) without any modification to the protocol of the kit. Diluted solutions of bacteriophage lambda DNA (Fermentas, Vilnius, Lithuania) were used to construct the calibration line. Fluorescence was measured in a Safire 2 spectrophotometer-fluorimeter (Tecan, Grödig bei Salzburg, Austria) at an excitation wavelength of 492 nm and emission wavelength of 520 nm. Amplifiablity of the isolated DNA was determined using universal eukaryotic real-time PCR [16].

Tab. 1. Oligonucleotides used in PCR.

Designation	Sequence (5'-3')	Target	Reference
pinbF	agcacttctcccgaacctca	wheat, barley, rye	13
pinbR2	gatggagcgatgtttcacaa	wheat, barley, rye	this article
pinb	FAM-ctcacagccgcccttccacca-TAMRA	wheat, barley, rye	13
TR03	tctgccctatcaactttcgatggta	eukaryotic	17
TR04	aatttgcgcgcctgctgccttcctt	eukaryotic	17
TRPb	FAM–ccgtttctcaggctccctctccggaatcgaacc-TAMRA	eukaryotic	16

Tab. 2. Results of PCR with wheat and barley cultivars, and barley and rye flour.

Cultivar	Country of origin	PCR result
Wheat Alexandria	Netherlands	+
Wheat Apache	France	+
Wheat Aztec	France	+
Wheat Baldus	Estonia	+
Wheat Brea	Czech Republic	+
Wheat Camp Remy	France	+
Wheat Charger	United Kingdom	+
Wheat Clever	United Kingdom	+
Wheat Garaboly	Hungary	+
Wheat Grommit	Denmark	+
Wheat Helle	Estonia	+
Wheat Hereward	Ireland	+
Wheat Korweta	Poland	+
Wheat Ludwig WW/03	Austria	+
Wheat Magnus	Germany	+
Wheat Petrana	Slovakia	+
Wheat Sani	Estonia	+
Wheat Shango	France	+
Wheat Soissons	France	+
Wheat Srpanjka	Croatia	+
Wheat Stava	Sweden	+
Wheat Super Žitarka	Croatia	+
Wheat Tommi	Germany	+
Wheat Žitarka	Croatia	+
Wheat Zorza	Poland	+
Wheat Zyta	Poland	+
Barley Dvoran	Slovakia	+*
Barley Forum	Slovakia	+*
Barley Pedant	Slovakia	+*
Barley flour	Germany	+*
Rye flour	Germany	+*

* – positive results at markedly higher c_T values.

Tab. 3. Results of PCR with plant species and other samples which do not contain coeliac disease-causing gluten.

Sample	PCR result
Amaranth	–
Broomcorn millet	–
Buckwheat	–
Cocoa	–
Maize	–
Oat	–
Pea	–
Potato	–
Rapessed	–
Rice	–
Soya	–
Sunflower	–
Almond	–
Brazil nut	–
Cashew nut	–
Hazelnut	–
Pistachio nut	–
Walnut	–

Polymerase chain reaction

PCR was performed in a volume of 25 µl. Each reaction contained 5 µl of template DNA, 1.5 U DNA polymerase HotStarTaq Plus (Qiagen, Hilden, Germany), 1× reaction buffer for the DNA polymerase, 2.5 mmol.l^{-1} $MgCl_2$, 200 µmol.l^{-1} dNTP mixture (Applied Biosystems, Foster City, California, USA), 200 nmol.l^{-1} of each primer and 200 nmol.l^{-1} of the probe (Tab. 1). Oligonucleotides were synthesized by Qiagen Operon, Köln, Germany. PCR was performed in ABI 7900 real-time PCR cycler (Applied Biosystems) or in Opticon 2 real-time PCR cycler (MJ Research, Waltham, Massachusetts, USA). The PCR programme consisted of the initial denaturation at 95 °C for 5 min followed by 60 cycles of denaturation at 95 °C for 15 s and annealing with polymerization at 60 °C for 60 s. Fluorescence was measured in channel 1 (FAM). In ABI 7900, threshold cycle values (c_T) were automatically calculated by the internal software of the instrument. In Opticon 2, c_T values were calculated by the internal software of the cycler using baseline subtraction and manual threshold setting at a fluorescence value of 0.035.

ELISA

Protein isolation and immunochemical detection of gliadin was carried out using RidaScreen Gliadin kit (R-Biopharm, Darmstadt, Germany) according to instructions for use included in the kit. The protein isolate was diluted 500× before applying to ELISA. Absorbance was measured in a Safire 2 spectrophotometer-fluorimeter (Tecan). Determined concentrations of gliadin were used to calculate the concentration of gluten using the formula provided in the instructions for use of the kit.

RESULTS AND DISCUSSION

A real-time PCR system of Alary et al. [13] was used, but a new reverse primer pinbR2 was designed using Primer Express software (Applied Biosystems) on the basis of the nucleotide sequence of the gene encoding for puroindoline b of wheat (*Triticum aestivum*; access number AB237167; National Center for Biotechnology Information, Bethesda, Maryland, USA; Tab. 1). The calculated length of the amplicon was 95 bp.

The 5'-nuclease real-time PCR consisting of primers pinbF, pinbR2 and the probe pinb was tested with a panel of plant species and cultivars. All the wheat, barley and rye samples were tested as positive by the assay, whereas all the other agricultural plant species were tested as negative

(Tab. 2, Tab. 3). The positive reactions for barley and rye were weaker than those for wheat cultivars (c_T values were markedly higher). This was probably caused by the fact that nucleotide sequences of the primers and of the probe matched perfectly only the nucleotide sequence of wheat puroindoline b gene, while a few mismatches were present with barley and rye nucleotide sequences. However, the high sensitivity of real-time PCR for wheat DNA can be taken as the most important parameter as long as wheat is the most common contaminant of "gluten-free" and naturally gluten-free food products.

The PCR system was applied to a series of four-fold diluted solutions of DNA isolated from the reference wheat flour. A quantitative response was determined and linear, basically identical calibration lines were obtained using both real-time PCR cyclers (Fig. 1). A detection limit of approx. 0.026 ng was determined, which corresponds to approx. 1.5 haploid genomes, given the genome size (1C) of *Triticum aestivum* is 17.67 pg [18]. In two out of four replicate analyses, a further dilution of wheat DNA (0.007 ng) could be detected by Opticon 2 instrument. This observation can be attributed to a stochastic character of PCR at low template copy numbers. We do not take this result to consideration regarding the detection limit of the method. The higher sensitivity of the method achieved by using Opticon 2 can be attributed to the higher sensitivity of the fluorimetric unit of the instrument, which is equipped with photomultiplier on the emission side.

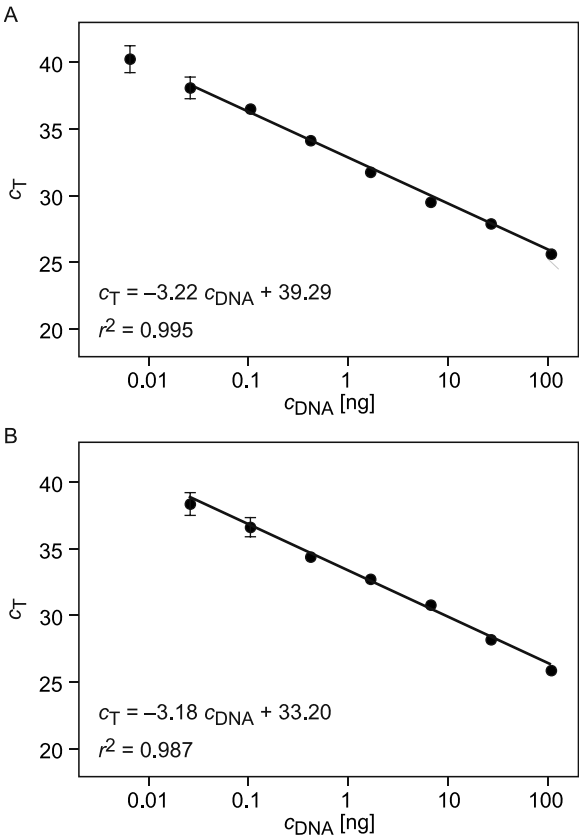

Fig. 1. Calibration line of threshold cycle *vs* DNA concentration.
A – Opticon 2 instrument, B – ABI 7900 instrument. Average values ± standard deviation of four replicate analyses are presented with the exception of data for the lowest DNA concentration (0.007 ng) where only two replicates produced valid results in Opticon 2 (A).

Tab. 4. Practical detection limit of real-time PCR and ELISA determined using model samples containing defined amounts of wheat flour.

Experiment No.	Detection limit for wheat [mg.kg^{-1}]	
	Real-time PCR	ELISA
1	200	200
2	200	500
3	200	100

The practical detection limit of the real-time PCR method was determined on model samples containing defined amounts of wheat flour in a "gluten-free" flour. A practical detection limit of 200 mg.kg^{-1} was determined. When the same model samples were analysed by ELISA, values ranging from 100 mg.kg^{-1} to 500 mg.kg^{-1} were determined (Tab. 4). These results demonstrate a better reproducibility of real-time PCR compared to ELISA. Response of real-time PCR in the range from 200 mg.kg^{-1} to 2000 mg.kg^{-1} was linear (Fig. 2).

Real-time PCR was further compared with ELISA at the analysis of "gluten-free" and naturally gluten-free food products. Results obtained by both methods were comparable. Out of 49 samples, 45 samples were found negative by both methods. Three samples, which were found positive by ELISA but conforming to the legally permitted gluten concentration, were positive also by real-time PCR. One sample was found positive only by real-time PCR (Tab. 5, Tab. 6). The three samples positive by ELISA were found to contain low levels of 1.1–2.9 mg.kg^{-1} of gluten and to contain wheat or barley or rye with threshold cycle values of real-time PCR of 38.70–37.51. The sample which was found positive only by real-time PCR had a threshold cycle value even higher (Tab. 6). These results demonstrate that real-time PCR was at least as sensitive as ELISA at the analysis of food samples.

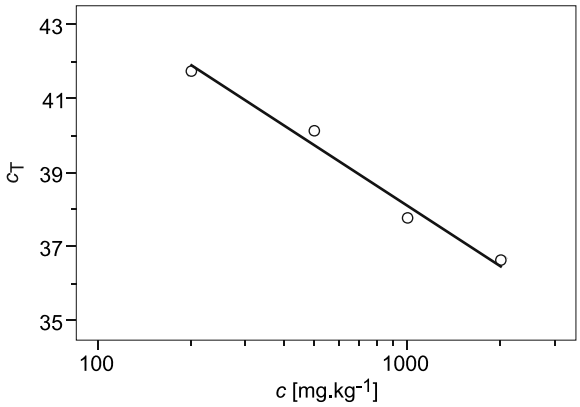

Fig. 2. Analysis of model samples containing defined amounts of wheat by real-time PCR.

Tab. 5. Results of the analysis of "gluten-free" food products by real-time PCR and by ELISA.

Sample	Country of origin	c_T	c_{gluten} [mg.kg^{-1}]
Biscuits 1	Italy	–*	–**
Biscuits 2	Italy	–	–
Biscuits 3	Slovakia	38.70	1.08
Biscuits 4	Slovakia	–	–
Biscuits 5	Slovakia	–	–
Biscuits with buck-wheat	Slovakia	–	–
Biscuits from extruded rice 1	Austria	–	–
Biscuits from extruded rice 2	Austria	–	–
Biscuits from extruded rice 3	Hungary	–	–
Breadcrumbs	Slovakia	–	–
Cake 1	Czech Republic	37.51	2.9
Cake 2	Slovakia	–	–
Cocoa wafers	Slovakia	–	–
Flour 1	Slovakia	–	–
Flour 2	Slovakia	–	–
Flour 3	Slovakia	–	–
Ham smoked	Slovakia	–	–
Lemon wafers	Slovakia	–	–
Müsli	Slovakia	–	–
Nut wafers	Slovakia	–	–
Pasta 1	Slovakia	–	–
Pasta 2	Italy	–	–
Pâté	Slovakia	–	–
Rice snack with chocolate	Czech Republic	–	–
Soya snack 1	Czech Republic	–	–
Soya snack 2	Czech Republic	–	–
Soya spread	Slovakia	–	–

Results of real-time PCR analyses are expressed as the threshold cycle value (c_T), results of ELISA analyses are expressed as gluten contents (c_{gluten}).
* – no positive amplification, ** – below the quantification limit.

Tab. 6. Results of the analysis of naturally gluten-free and other food products by real-time PCR and by ELISA.

Sample	Country of origin	c_T	c_{gluten} [mg.kg^{-1}]
Amaranth flour	Czech Republic	–*	–**
Amaranth pasta	Czech Republic	–	–
Amaranth risotto (dry mixture)	Slovakia	–	–
Broomcorn millet	Slovakia	–	–
Candies	Czech Republic	–	–
Cheese	Czech Republic	–	–
Chicory root, roasted	Slovakia	–	–
Chocolate	Czech Republic	–	–
Chocolate with coconut	Czech Republic	–	–
Chocolate with pistachio flavouring	Czech Republic	38.99	–
Corn flakes	Slovakia	–	–
Ice cream	Czech Republic	–	–
Ketchup	Slovakia	–	–
Maize flour	Czech Republic	38.63	2.2
Margarine spread	Czech Republic	–	–
Mayonnaise sauce	Slovakia	–	–
Mustard condiment	Slovakia	–	–
Rice pap, instant	Czech Republic	–	–
Vinegar	Slovakia	–	–
Yoghurt 1	Germany	–	–
Yoghurt 2	Slovakia	–	–
Yoghurt 3	Slovakia	–	–

Results of real-time PCR analyses are expressed as the threshold cycle value (c_T), results of ELISA analyses are expressed as gluten contents (c_{gluten}).
* – no positive amplification, ** – below the quantification limit.

CONCLUSIONS

The presented PCR method is useful for sensitive and selective detection of coeliac disease-causing gluten-containing cereals in food samples. The method is at least as sensitive as ELISA. It is relatively straightforward and fast, the analysis can be performed in one working day.

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