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Evaluation of a Real-Time PCR to Detect Coeliac-Toxic Components and Comparison to the ELISA Method Analysing 35 Baby Food Samples

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Introduction

Coeliac disease is a genetic, immunologically mediated small bowel enteropathy that can cause different gastrointestinal, metabolic, musculoskeletal and neuropsychiatric symptoms (1). The patients concerned cannot include wheat, barley or rye in their strict gluten-free diet. Mainly newborns should not be exposed too early in life to these coeliac-toxic components (2, 3). Breast feeding practice has the positive effect by delaying the time point of the gluten introduction and thus results in a prolonged latency time between gluten introduction and onset of the disease (4). Studies made among breastfed and non-breastfed infants showed that human milk protects against infections via the secreted IgA antibodies (5). Such an enhanced immunological function could also explain why breastfeeding may protect against diseases like coeliac disease and allergy (6). Therefore it is very important that babyfood products are gluten-free and no coeliac-toxic component is consumed by babies within the first 4–6 months.

A quantitative-competitive PCR-system (QC-PCR) to detect contamination of wheat, barley or rye in gluten-free food (7) was developed. Since the QC-PCR can be substituted with the LightCycler™ technology (e.g. in serology and microbiology (8, 9)) the present study evaluates the potential for real-time PCR for wheat, barley and rye. An advantage of the real-time assay is the overall reduced time of the whole process to 20–30 minutes by the use of air instead of thermal blocks and a high surface-to-volume capillary tube format. Second, the results can be followed in

real-time as the reaction proceeds. Third, the closed capillary system eliminates the risk of contamination. Forth, the handling is less laborious and it does not require any post-PCR manipulations.

In this study, 35 most frequently consumed baby food products for newborns and infants, all labelled as gluten-free and hypoallergenic were analysed.

The Codex Alimentarius (10) defines a gluten-free food to contain less than 100 ppm gliadin analysed by ELISA (enzyme linked immuno sorbent assay). Thus the allowed limit for gluten-free food is 10 mg gliadin/100 g dry weight. Since wheat contains 5 % gliadin, the limit of 10 mg gliadin corresponds to 200 mg of wheat. This means that not more than 200 mg of wheat is allowed per 100 g of a product i.e. to 0.2 % of the product. With the presented real-time PCR it was checked if there exists babyfood products which have more than 0.2 % wheat DNA in their background DNA (above the allowed limit).

Enzyme-linked immunosorbent assays (ELISA) use mono- or polyclonal antibodies against gliadins. The only assays which have been applied in interlaboratory tests and are commercially available are based on a monoclonal antibody against a part of the ω-gliadin fraction (11–13). The disadvantage of the ELISA is that various cultivars have differences in the proportions of ω-gliadins, resulting in systematic errors. Though protein contents and the proportions of ω-gliadins are similar, the affinity of the antibodies to different gliadin standards can strongly vary. By comparing several ELISA-test kits, it has been shown that the measured gliadin contents varied greatly. Therefore, none of the present methods allows the determination of the true gliadin content in gluten-free foods. There is still a requirement for a sensitive and fast assay to screen different foods on the market.

In a first step the DNA quality was controlled on an agarose gel after extraction of the nucleic acids from different processed baby foods. Then the amplificability of DNA was checked by a plant-specific PCR (14) and real-time PCR was applied to the baby food samples in order to determine the content of wheat DNA. In parallel, gluten from wheat, barley or rye was analysed with the ELISA method. Finally, results of both methods were compared to each other. Real-time PCR and ELISA yielded identical results for all commercial products indicating a comparable sensitivity of both methods. Out of the 35 baby-foods, 34 were below the allowed limit of 0.2 % wheat DNA or 100 ppm gliadin (10 mg gliadin/100 g dry weight) and could be considered as safe for babies.

Materials and methods

DNA extraction

300 mg of homogenized sample were incubated with 860 µl of TNE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA and 1 % (w/v) SDS), 100 µl of 5 M guanidine hydrochloride and 40 µl of 20 mg/ml proteinase K (Merck, Darmstadt, Deutschland) on a thermomixer (Eppendorf, Hamburg, Germany) at 58° C

for at least 3 hours. Co-extracted RNA was removed by 5 µl of RNase A (10 mg/ml) (Promega, Madison, WI, USA) at 58° C for 5 min. The solution was then purified using the Wizard protocol (Promega). Finally, the DNA was eluted in 100 µl of 10 mM Tris-HCl, pH 9.0 DNA, concentrations were determined spectrophotometrically using a GeneQuant II RNA/DNA Calculator (Pharmacia, Uppsala, Sweden).

PCR

PCR was carried out in a 50 µl reaction volume for plant-specific PCR (14) containing 200 ng of template DNA, 2.5 mM of $MgCl_2$, 0.5 µM of each oligonucleotide primer TAB03 and TAB04 (Microsynth, Balgach, Switzerland) (table 1), 200 µM of each dNTP (Promega), 1× reaction buffer (Promega), 2 µg/ml of bovine serum albumin and 1 unit of *Taq* DNA polymerase (Promega). PCR was performed on a Progene thermocycler (Techne, Princeton, NY, USA) as follows: initial denaturation at 94° C for 3 min, 35 cycles at 96° C for 20 sec, 54° C for 40 sec, and 72° C for 40 sec, and extension at 72° C for 3 min. PCR products were analyzed on 1.5 % (w/v) agarose gels.

Real-time PCR with SYBR Green I

Reactions on the LightCycler™ with DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) were performed in 20 µl volumes containing 50 ng of template DNA, 3 mM of $MgCl_2$, 0.5 µM of each oligonucleotide primer WBR11 and WBR13 (table 1) and 0.5 µM LightCycler DNA Master SYBR Green I (Roche). The temperature profile was as follows: initial denaturation at 95° C for 30 sec, followed by 60 cycles of denaturation at 95° C for 0 sec (immediate cooling), annealing at 55° C for 5 sec, and final extension at 72° C for 10 sec. Melting curve analysis (to control correct amplification) and cooling were done according the instructions of the manufacturer.

Real-time PCR with hybridisation probes

The standard temperature profile for these assays included in the 20 µl reaction volume 100 ng of template DNA, 4 mM of $MgCl_2$, 0.5 µM of each oligonucleotide primer WBR11 and WBR13, 0.4 µM of each Hybridisation probe (TIB MOLBIOL, Berlin, Germany) WBR-LC1 and WBR-LC2 (table 1) and 2 µl mastermix (LightCycler-DNA Master Hybridization Probes, Roche). The temperature profile was as follows: initial denaturation at 95° C for 30 sec, followed by 60 cycles of denaturation at 95° C for 0 sec, annealing with fluorescence monitoring at 58° C for 10 sec, and extension at 72° C for 10 sec.

Calibration of standard curves

50 ng of DNA containing 50, 20, 10, 5, 2, 0.2 and 0.02 % wheat DNA with a background of soy DNA were amplified. Reproducibility was checked through

Table 1
Primers used in this work

Name	*Orientation*	*Sequence (5'-3')*	*Reference*
TAB03	sense	CGAAATCGGTAGACGCTACG	(11)
TAB04	antisense	GGGGATAGAGGGACTTGAAC	(11)
WBR11	sense	GGTAACTTCCAAATTCAGAGAAAC	(7)
WBR13	antisense	TCTCTAATTTAGAATTAGAAGGAA	(7)
WBR-LC1	sense	AGACTCAATGGAAGCTGTTCTAACGA	(this work)
WBR-LC2	sense	Red640-CGAGTTAATTACGTTGTGTTGTTAGTGG	(this work)

WBR = wheat-barley-rye detection system
TAB = plant system

intra- and interassays. In the intra-assay the samples of the standard curve were analysed in parallel four times in the same experiment and the variation was then calculated for every value. The intra-assay was repeated five times. In the interassay the samples of the standard curve were analysed on subsequent days and the variation was evaluated for every value. Conditions for real-time PCR were as described above.

After PCR cycling, the LightCycler™ software converts the raw data to the amount of DNA. The first step is performed by setting a threshold value on a fluorescence level, at which the fluorescence development reflects that the PCR is in the log-linear phase. The software then calculates the logarithmic values for all of the data points that are above this noise band. By interpolating a straight line through an user-defined number of data points above this threshold value, the software then determines the points of intersection with the noise band for all of the standards. These points of intersection, which are referred to as crossing points (cycle numbers) in the software, are plotted against the logarithm of the concentration. The concentrations of target sequence in the standards and in the samples with unknown concentrations are obtained by comparing the crossing points for the samples with the crossing points of the standards.

Initial experiments with standard curves and reference samples were analyzed both with SYBR Green and HybProbes and found to be identical (data not shown). Therefore, the cheaper SYBR Green was used for further experiments.

Determination of baby food samples

All products were extracted and analysed in duplicates. The content of wheat DNA was evaluated by real-time PCR to be above or below 0.02 % and 0.2 %.

Gliadin-ELISA

Gliadin extraction and analysis were performed as recommended in the distributor's manual of the Ridascreen Gluten kit (R-Biopharm, Darmstadt, Germany) (13).

Results and Discussion

Coeliac disease commonly appears during the first two years of life, after several months of wheat protein ingestion (15). The treatment of coeliac patients consists of a strict gluten-free diet.

The aim of this study was to apply the existing WBR (wheat, barley and rye)-quantitative-competitive (QC)-PCR system (7) to a real-time PCR format and to screen different baby food products. Since the ELISA assay is still the only established in the Codex Alimentarius (10) for testing gluten-free products, the presented data should indicate if the real-time PCR might be an additional method to be used.

The QC-PCR system was adapted to the LightCycler™ with respect to $MgCl_2$ concentration and program profile. The specificity of the WBR-system has been

tested in the previous work (7). The hybridization probes WBR-LC1 and WBR-LC2 and primers WBR11 and WBR13 were defined on the intron of the chloroplast *trnL* gene (14). The oligonucleotides used for PCR amplify a 201 bp fragment for wheat and rye and for barley a 196 bp long fragment. The LightCycler™ Red640 labelled probe (WBR-LC2) is located 3 bp downstream of the fluorescein labelled probe (WBR-LC1) and 6 bp upstream of the lower primer WBR13. The samples for the standard curves from wheat in soy DNA ranged from 50, 20, 10, 5, 2, 0.2 to 0.02 %. All PCR reactions of the standards were performed in quadruplicate; the intra-assay variation was <10 %, and interassay variation was <15 % (data not shown).

The different baby food samples are subdivided into infant formula samples (from the first day of life; samples #1–11) and such for nurslings (from the 4th month; samples #12–35; see table 2). Baby food products are strictly regulated in most countries. Infant formula samples are based on whey, vegetable fat and oil, skim milk powder, maltodextrin, lactose, milkproteins, minerals, vitamins, starch and emulsifiers. Starch was derived from rice-, maize- or potato but not from wheat. The products were all labelled as gluten-free and hypoallergenic.

All of the chosen infant formula samples #1–11 (table 2) resulted in DNA-amounts from 3000 to 9000 µg/100 g. Samples #1–8 showed a signal in plant-specific PCR, which means that plant DNA was present and amplifiable. In the mentioned products lecithin or starch of potato or rice was added. With respect to WBR-real-time PCR, wheat DNA content of this samples was below 0.02 %. Samples #9–11 produced no amplicon in plant-specific PCR. Reasons for this were that samples #9–11 contain no vegetable ingredients. However, this does not completely exclude the presence of possible contaminations with coeliac-toxic components in these products.

For samples #1–11, gliadin-content in the ELISA was below 10 ppm, which indicates that all tested products for newborns could be considered as safe. Samples #12–35 should give an intake with nutrients for an optimal growth in the first year of life of the babies. They included paps made of vegetables, fruit or milk. All of the chosen products resulted in DNA-amounts from 2000 to 14 000 µg/100 g. Samples #12–31 resulted all in a signal in plant-specific PCR. WBR-real-time PCR yielded for all samples (except for sample #25) wheat DNA percentages of less than 0.02 %. Sample #25 had a wheat DNA content of 0.2–0.02 % but still in the allowed limit of the Codex Alimentarius. Concerning the ELISA results, evaluation of these samples with real-time PCR revealed that no product was above the limit value of 100 ppm or 0.2 % wheat DNA, respectively. In the ELISA, samples #25 to #29 showed that the gliadin contents were between 10–100 ppm, which indicates that these samples are gluten-free, too. Samples #31 was both positive in real-time PCR as well as in the ELISA. Sample #31 was extracted twice in duplicates in order to exclude contamination. Samples #32–35 were also prepared twice in duplicates. Plant-PCR never resulted in a signal even though vegetable ingredients were present. Reasons for this

Table 2
DNA-amount, qualitative plant-PCR, quantitative real-time PCR and ELISA results of 35 baby food samples (+, detected PCR product, –, no PCR product, •, no real-time signal). 0.2% wheat DNA corresponding to 100 ppm. 1 ppm means 0.1 mg gliadin/100 g food

Infant formula samples	*dsDNA µg/100 g*	*Plant-PCR*	*WBR REAL-TIME PCR*	*ELISA*
#01	4750	+	<0.02%	<10 ppm
#02	5916	+	<0.02%	<10 ppm
#03	3866	+	<0.02%	<10 ppm
#04	3066	+	<0.02%	<10 ppm
#05	7565	+	<0.02%	<10 ppm
#06	5266	+	<0.02%	<10 ppm
#07	7915	+	<0.02%	<10 ppm
#08	4999	+	<0.02%	<10 ppm
#09	3099	–	•	<10 ppm
#10	4232	–	•	<10 ppm
#11	8815	–	•	<10 ppm
Baby food samples for nurslings (4th month)	*dsDNA µg/100 g*	*Plant-PCR*	*WBR REAL-TIME PCR*	*ELISA*
#12	6782	+	<0.02%	<10 ppm
#13	2066	+	<0.02%	<10 ppm
#14	2383	+	<0.02%	<10 ppm
#15	4666	+	<0.02%	<10 ppm
#16	5149	+	<0.02%	<10 ppm
#17	2183	+	<0.02%	<10 ppm
#18	14631	+	<0.02%	<10 ppm
#19	3499	+	<0.02%	<10 ppm
#20	3716	+	<0.02%	<10 ppm
#21	7765	+	<0.02%	<10 ppm
#22	3816	+	<0.02%	<10 ppm
#23	4949	+	<0.02%	<10 ppm
#24	2766	+	<0.02%	<10 ppm
#25	7799	+	0.02–0.2%	10–100 ppm
#26	7049	+	<0.02%	10–100 ppm
#27	4199	+	<0.02%	10–100 ppm
#28	2033	+	<0.02%	10–100 ppm
#29	5899	+	<0.02%	10–100 ppm
#30	5399	+	<0.02%	<10 ppm
#31	2999	+	>0.2%	>100 ppm
#32	8949	–	•	<10 ppm
#33	2916	–	•	<10 ppm
#34	6582	–	•	<10 ppm
#35	2549	–	•	<10 ppm

were probably processing steps during manufacturing of the products which degraded nucleic acids or substances which inhibited the PCR-reaction. In this case ELISA should be used to check for coeliac-toxic compounds.

It can be concluded that real-time PCR for the detection of gluten-free products for coeliac patients presents an additional method to the established ELISA method. There exists the possibility to differentiate in the presence of the coeliac-toxic components between DNA- or protein content by the use of PCR or/and ELISA. In the case of wheat free products containing gliadin which would be an indicator that purified gliadin is used as an ingredient the ELISA method is a good detection method. And in the presence of samples with gluten-free wheat starch the PCR method yields informations about the DNA amount of added wheat, barley or rye. So the origin of contamination can be better identified.

The results in this study (real-time PCR and ELISA) showed to be identical with respect to the limit value. Samples #26–29 resulted in the ELISA in 10–100 ppm which would indicate a wheat DNA content of 0.02–0.2 % in real-time PCR. However, WBR-real-time PCR yielded for these samples wheat DNA percentages of less than 0.02 %. This can be explained by the fact that the values in the ELISA were just above 10 ppm (data not shown). For three products (samples #32–35) it was not possible to amplify plant DNA. Processing procedures during manufacturing and inhibitors in food could influence PCR reaction. It should be mentioned that contamination resulting from wheat-containing products, which are manufactured in the way to contain no amplifiable DNA, could not be detected with this method. This problem exists already since the methodology of PCR is available. But this problem is also seen in the ELISA since antibodies could not recognize food proteins which are degraded or have an altered structure of the binding site.

The WBR-PCR-system described in this study is based on the amplification of the intron of the chloroplast *trnL* gene. Different vegetables and tissues like leafs, tubers, grains etc. have different amounts of chloroplasts resulting in varying amounts of DNA which may influence quantitation of real-time PCR. Therefore, results obtained from products with a huge part of different vegetable ingredients and tissues should be carefully interpretated.

However, in the determination of gluten-free products mainly cereals such as maize, millet, rice and soy (flour or processed products like bread, pasta, pastry and flakes) have to be analysed. It is not known enough about the variation of chloroplast copies between wheat cultivars. If there exist differences, theoretically, it should be made a standard for quantitation for each cultivar. But since it is known which wheat species is manufactured in the food samples, this would not be useful.

Thus, real-time PCR could be regarded as a safe and quick method in the evaluation of gluten-free products. Out of the 35 chosen products 35 samples showed equal results with both methods, indicating that the presented real-time PCR is a valid addition to the ELISA for the detection of gluten in food samples.

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Zusammenfassung

Zöliakie-Betroffene müssen ihr Leben lang eine strikt glutenfreie Diät einnehmen, um die vielfältigen Symptome vermeiden zu können. Damit dies eingehalten werden kann, müssen die speziell mit dem glutenfrei gekennzeichneten Lebensmittel überprüft werden. Die bis heute einzig im Codex Alimentarius etablierte Untersuchungsmethode stellt der ELISA dar. In dieser Studie wird ein bereits entwickeltes quantitativ kompetitives PCR-System, welches weizen-, gerste- und roggenspezifisch ist, auf die neue real-time PCR-Technologie adaptiert. Anhand von real-time-PCR und ELISA wurden 35 Babynahrungen untersucht. Die erhaltenen Resultate stimmen gut miteinander überein, was zeigt, dass die real-time-PCR eine ergänzende Methode zum ELISA darstellt.

Résumé

Les personnes qui souffrent de la maladie de coeliakie doivent observer durant leur vie un régime sans gluten. A cette fin, les aliments spéciaux déclaré «sans gluten» doivent être contrôlés. A ce jour, l'unique méthode recommandée par le Codex Alimentarius est l'analyse ELISA. Une méthode existante basée sur l'analyse PCR quantitative compétitive spécifique au blé, à l'orge et au seigle a été adoptée pour développer la nouvelle méthode real-time PCR. 35 produits pour bébé ont été examinés à l'aide des méthodes real-time PCR et ELISA. Les résultats obtenus ont été concordants, la méthode real-time PCR peut donc être utilisée en complément de l'analyse ELISA.

Summary

Coeliac patients have to adhere to a lifelong strict diet to prevent the various symptoms. In order to realize this diet, food labelled as gluten-free have to be analyzed. The ELISA assay is still the only established test stipulated by the Codex Alimentarius. In this study an existing wheat-, barley- and rye-specific quantitative competitive PCR-system (QC-PCR) is adapted to the new real-time PCR technology. 35 baby food products were analysed by real-time PCR and ELISA. The results correlated well to each other indicating that real-time PCR can be applied as additional method to ELISA.

Key words

Quantitative PCR, Real-time PCR, ELISA, Gluten-free baby food

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