



A polymerase chain reaction directed to detect wheat glutenin: Implications for gluten-free labelling

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ABSTRACT

Gluten enteropathy or celiac disease (CD) is treated by strict adherence to gluten-free diet for life. Trace amounts of wheat in food from farm to table manifests as a major risk to these individuals. A qualitative polymerase chain reaction method was developed by selectively amplifying a 135 bp fragment of the glutenin gene to detect wheat DNA in a plethora of raw and heat processed foods. The limit of detection was 21.5 pg of DNA. The absence of amplification in other cereals such as oat, rye, barley and maize renders this method exclusive for detection of wheat. The detection of wheat DNA in thermally processed foods by this method, despite extensive DNA fragmentation, evinces the suitability and applicability of the method for labeling gluten-free foods. This method complements the immunochemical methods toward addressing food safety in CD patients and wheat allergics.

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1. Introduction

Celiac disease (CD), the most common food sensitive enteropathy in humans (Branski, Fasano, & Trincone, 2006) is a chronic inflammatory disorder of the upper small intestine. CD patients suffer an inappropriate immune response to proteins of all *Triticum* species and homologs from other cereals such as barley, rye and possibly oats (Kasarda, 2001). Lifelong diets, exclusive of gluten are therefore essential for small bowel recovery and to avoid complications such as intestinal lymphoma (Holmes, Prior, Lane, Pope, & Allan, 1989). This translates into excluding all forms of wheat (*Triticale* sp.), triticale, rye, barley and oat products as the only remedy (Kasarda & D'Ovidio, 1999). A very special category of food products labeled "gluten-free" in accordance with the specifications of the Joint FAO/WHO Food standard Programme Codex Alimentarius Commission (2006) (Draft revised standard for gluten free foods. *Alinorm*, 07/03/26) is the choice.

Wheat and wheat based products are among the staples of a large percentage of the world population. Lifestyle changes have resulted in packaged and convenience foods mainly "bakery products" which often comprise of wheat or other gluten containing cereals. Cross contamination of packaged and traditional gluten-free and wheat-free products can occur at any stage from farm through food processing. Cereal and cereal fractions are ubiquitous additives in the processed-food industry. Therefore it is necessary that all the products and ingredients be analyzed to

comply with gluten-free labeling. Wheat gluten is composed of both gliadins and glutenin. Currently the analytical method for evaluating gluten-free is based on immunochemical detection of gliadin as specified by Joint FAO/WHO food standard programme of Codex Alimentarius Commission (2006). The available enzyme linked immune-sorbent assay (ELISA) is based on a monoclonal antibody against an ω -gliadin fraction (Skerritt & Hill, 1991; Windemann, Fritschy, & Baumgartner, 1982). These methods also show cross reactivity to both barley and rye gluteins (Lardizabal, Niemann, & Hefle, 2002). Very recently a sandwich and competitive ELISA based on monoclonal antibodies to an exceptionally immunoreactive 33-mer peptide of α -2 gliadin were developed to detect the toxic peptide in hydrolysed foods at <0.5 ppm gliadin (Moron et al., 2008). The effect of extensive protein denaturation during the technological processes involved in food manufacture leads to uncertainty and insufficient immuno-specificity (Denery-Papini, Nicolas, & Popineau, 1999). The stability of DNA and the use of highly specific and sensitive polymerase chain reaction (PCR) techniques can provide quantitative molecular markers to detect gluten and differentiate wheat gluten from the other homologous cereal gluteins. PCR is also the method of choice in food forensics for detection of (a) genetically modified organisms (Hubner, Studer, & Lüthy, 1999; Hupfer, Hotzel, Sachse, Moreano, & Engel, 2000; Studer, Dahinden, Lüthy, & Hubner, 1997; Studer, Rhyner, Lüthy, & Hubner, 1998), (b) bread wheat in pasta products (Bryan, Dixon, Gale, & Wiseman, 1998), (c) food pathogens (Sidhu, Holmes, Allison, & Peck, 1999) and (d) identification of fish and game animal species used in various meat products (Meyer, Candrian, & Lüthy, 1994; Meyer, Höffelein, Lüthy, &

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Candrian, 1995). A quantitative competitive PCR method to detect wheat, barley or rye in gluten free food for CD patients has been constructed based on an amplification of the gibberelin gene (Dahinden, Von Buren, & Lüthy, 2001) and an intra-laboratory validation carried out on bakery products (Olexova, Dovicovicova, Svec, Siekel, & Kuchta, 2006). This method however does not distinguish between the cereals wheat, barley and rye. A real time PCR method based on melting curve analysis discriminates between the different cereals. When comparing samples that have been subjected to different degrees of processing the real time PCR quantitation is not reliable (Sandberg, Lundberg, Ferm, & Yman, 2003).

In this study a PCR based method directed exclusively at detecting wheat gluten to ensure the safety of “gluten-free” labeling for not only CD patients but also wheat allergics is described. The GenBank sequences of the cereal glutenins were aligned and specific primers designed to amplify a wheat DNA sequence. The method is applicable to convenience and traditional foods that have been thermally processed viz. oven baked, deep fried, extruded and griddle cooked. This method complements the widely used ELISA method. The detection limits achieved, equivalent to 21.5 pg of wheat DNA is below the Codex Alimentarius limits for gluten in gluten-free foods.

2. Materials and methods

2.1. Cereal samples and food products

The wheat varieties *Triticum aestivum* (bread wheat), *Triticum durum* (durum wheat) and *Triticum dicoccum* (emmer wheat) were collected from the Department of Flour Milling and Baking Technology, Central Food Technological Research Institute Mysore, Karnataka. Whole grains of barley (*Hordeum vulgare*), rye (*Secale cereale*), oats (*Avena sativa*), corn (*Zea mays*), rice (*Oryza sativa*) and soya (*Glycine max*) were obtained locally. Whole-wheat flour, refined wheat flour, semolina, bread, pizza-base, patties, cake, rusk, biscuits, instant noodles, infant formula, wheat flakes, vermicelli, rice sevai, rice flakes, and puffed rice were purchased at the local supermarket. Traditional Indian foods like chapatti (unleavened bread), puri (deep-fried) and samosa (deep-fried dumpling) were prepared with wheat flour according to Prabhaskar, Manohar, and Gowda (2002). Ragi and rice noodles were from the Department of Grain Science and Technology of this institute.

2.2. Chemicals

Taq PCR core kit, RNaseA and Proteinase K were obtained from Qiagen GmbH, Hilden, Germany. Agarose was from Amresco, Solon, Ohio, USA. Cetyl trimethylammonium bromide (CTAB), ethidium bromide and α -amylase were procured from Sigma Aldrich, USA. 6 $\times$ mass loading dye and 100 bp DNA ladder were obtained from Fermentas Inc, Maryland, USA.

Genomic DNA extraction: All the cereal grains and seeds were ground in a grinder to obtain a fine powder. The finished food and commercial products were defatted with hexane and homogenized to a fine powder. DNA was extracted following the CTAB method (ISO/TC34WG7N31, 2001). The DNA concentration was determined by UV absorption at 260 nm (1 absorbance unit = 50 μ g mL⁻¹ of ds DNA). The purity of the extracted DNA was assessed by the 260/280 nm absorption ratio.

Oligonucleotides: The sense and antisense primer sequences were designed using Primer Express Version 2.0 (Applied Biosystems, USA). The oligonucleotides synthesized by Sigma Genosys, India were stored as 0.1 mM solution at -20 °C until used. The oligonucleotides were diluted to (10 pmols μ L⁻¹) prior to use.

PCR: PCR was carried out in a 50 μ L reaction volume using a Gene Amp[®] PCR system 9600 (Applied Biosystems, USA). Each reaction contained 75 ng of template DNA, 1 $\times$ reaction buffer (Qiagen), 200 μ M of each dNTP, 0.9 units of Taq DNA Polymerase (Qiagen) and 10 pmols of each primer (gluF-5'GGGCCAATGTG-TGTTTCCA and gluR-5'GCAATGGAAGTCATCACCT). The following thermal cycling profile was programmed for the PCR reaction: 1 cycle of 2 min denaturation at 95 °C, 25 cycles 30 s at 94 °C, 40 s at 64 °C, 60 s at 72 °C and a final extension at 72 °C for 3 min. The plant specific 18S rDNA was amplified to evaluate the amplifiability of the extracted DNA (Allmann, Candrian, Hofelein, & Lüthy, 1993). The PCR products were analysed on 2.0% (w/v) agarose gels and stained with ethidium bromide. The reproducibility of the PCR patterns was verified by repeating the amplification in quadruplicate. To determine the Limit of detection (LOD) wheat genomic DNA solutions were serially diluted to 215, 21.5, 2.15, 0.215, 0.0215 and 0.00215 ng/ μ L. One microliter of wheat genomic DNA was then added to each PCR reaction.

PCR product validation: PCR products were purified using the Gene-Elute[™] PCR cleanup kit (Sigma, USA) following the manufacturer's protocols. The sequencing reaction was performed using gluF/gluR primers and the BigDye[®] terminator v 3.1 cycle sequencing kit (Applied Biosystems, USA), products purified using Dye Ex[™] 2.0 spin kit (Qiagen, Germany) and sequenced using an ABI 310 DNA Genetic Analyzer (Applied Biosystems, USA).

2.3. Eukaryotic specific PCR

The PCR mixture contained 1 $\times$ PCR buffer, 200 μ M dNTPs, 0.6 units of Taq Polymerase, 20 pmol each of the sense and antisense primer (Allmann et al., 1993) and 75 ng of template DNA. The following thermal cycle conditions were used: denaturation of DNA at 95 °C for 4 min 30 s, followed by 30 cycles of denaturation at 95 °C for 46 s, annealing at 65 °C for 1 min 26 s and, extension at 72 °C for 1 min 25 s. A final extension step was performed at 72 °C for 3 min 15 s.

2.4. Results and discussion

Unidentified wheat presence in the food supply manifests as a health risk to both the wheat allergic and CD patients. Their diet therefore requires careful scrutiny of food labels for the presence of wheat and other grains including rye, barley and oats. Consequently products need to be labeled either as “wheat-free” or “gluten-free”. The primary aim of this study was to develop an end point DNA based method to detect wheat in both raw materials and processed foods. Common food items including breads, pastries, pasta and traditional Indian foods subjected to various degrees of thermal processing were evaluated.

2.5. Selection of wheat DNA sequence for amplification

The premise of a successful PCR based detection method is the amplification of a specific target DNA sequence. Water/salt insoluble gluten (containing gliadin and glutenin) represents 80% of all wheat proteins. Glutenin, the major storage proteins of wheat are further classified into the high molecular weight (HMW: 70–90,000 Da) and low molecular weight (LMW: 20–45,000 Da) subunits (D'Ovidio and Masci, 2004). The LMW subunits are polymeric protein components of the endosperm and comprise about 40% of the total wheat gluten fraction (Zhang, Gianibelli, Rampling, & Gale, 2004). Wheat, rye and barley of the *Triticeae* tribe are phylogenetically closely related species. The LMW glutenin gene sequence of wheat (U86029), rye (AJ314782) and barley (CV053693) were retrieved from GenBank (Fig. 1). *In silico* analyses (using ClustalW) was used to identify sequence divergence. The

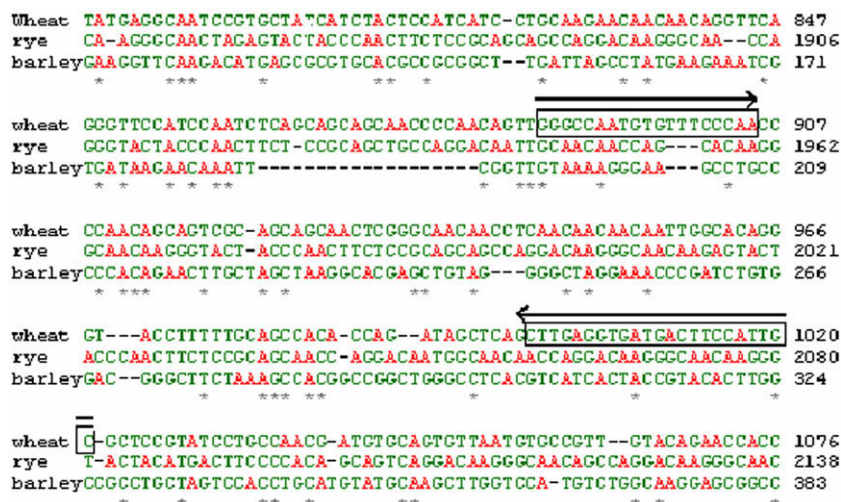


Fig. 1. Multiple alignment of LMW glutenin gene sequences of wheat (U86029), rye (A314782) and barley (CV053693) using ClustalW. The sequence of the sense primer gluF and complementary sequence to the antisense primer gluR are boxed.

region 887–1021 (wheat DNA sequence) showed an identity of <18.5% and therefore was chosen as the target amplification sequence. Two primers gluF and gluR were designed to amplify this sequence

2.6. Evaluation of the primers

The specificity of the primer pair (gluF/gluR) to amplify a 135 bp fragment was evaluated using genomic DNA extracted from wheat (*T. aestivum*, *T. durum* and *T. dicoccum*) and other cereals: maize (*Z. mays*), rice (*O. sativa*), finger millet (*Eleusine coracana*), proso millet (*Panicum miliaceum*), oat (*A. sativa*), bajra (*Pennisetum glaucum*), rye (*S. cereale*) and barley (*H. vulgare*). Positive amplification of a 135 bp fragment was observed in all the three varieties of wheat viz. *T. aestivum*, *T. durum* and *T. dicoccum* (Fig. 2, Lanes 6–8). No amplification was observed when DNA from phylogenetically similar rye, barley and other cereals were used as template (Fig. 2, Lanes 3–5 and 9–13). The absence of amplification due to the presence of PCR inhibitors was negated by an additional eukaryotic specific amplification. The primer pair TRO3/TRO4 was used to amplify a 137 bp region of the highly conserved repeat unit within

18S rDNA of eukaryotes (Allmann et al., 1993). A 137 bp amplicon was observed for rye and barley (Fig. 2, Lanes 14 and 15) and other cereals (results not shown) indicating that the DNA was of amplifiable quality and the absence of amplification with the primer pair gluF/gluR was not due to the presence of inhibitors. These results advocate that the method is specific for detection of wheat DNA.

2.7. Limit of detection (LOD)

The LOD of a PCR is defined as the lowest concentration of genomic DNA that can be reliably detected by the qualitative methods but not necessarily quantified. PCR amplification using template DNA ranging from 215 ng to 0.2 pg was carried out to determine the LOD. The results indicate that the minimum DNA concentration required for amplification was 21.5 pg (Fig. 3). Taking into consideration the wheat genome size of 16.5–19.5 pg per haploid genome (Arumuganathan & Earle, 1991), the LOD corresponds to an average of 1.1–1.3 DNA copies. Clinical sensitivity differs among wheat allergy and CD patients. Although the Codex Alimentarius limit for gluten is 20 mg per 100 g, the debate regarding the maximum permitted amount of gluten that patients with CD can safely tolerate continues. Some individuals cannot tolerate even trace amounts of gluten, whereas others appear to tolerate large transgressions. DNA fragmentation during unit operations in food manufacture reduces the copy number of amplifiable DNA. The LOD of this meth-

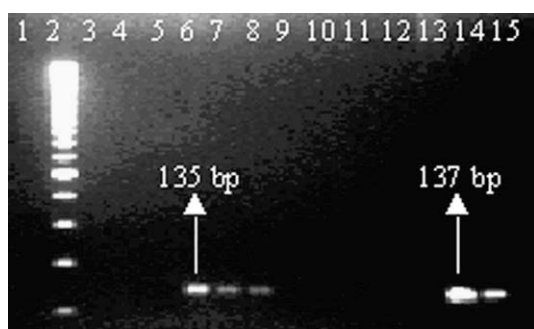


Fig. 2. Agarose gel electrophoresis profile showing the specificity of the primer pair gluF/gluR. Lane 1, 100 bp ladder; Lane 2, 135 bp band; Lane 3, 137 bp band; Lane 4, 135 bp band; Lane 5, 137 bp band; Lane 6, 135 bp band; Lane 7, 137 bp band; Lane 8, 135 bp band; Lane 9, 137 bp band; Lane 10, 135 bp band; Lane 11, 137 bp band; Lane 12, 135 bp band; Lane 13, 137 bp band; Lane 14, 135 bp band; Lane 15, 137 bp band.

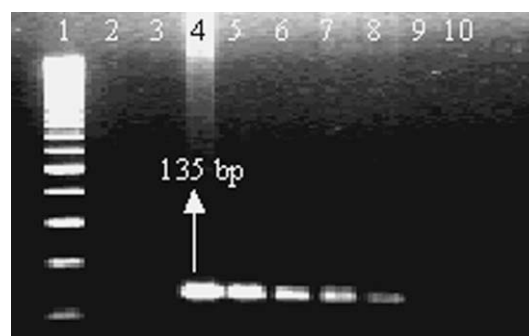


Fig. 3. (a) Agarose gel electrophoresis profile to determine the LOD. Lane 1, 100 bp ladder; Lane 2, 135 bp band; Lane 3, 137 bp band; Lanes 4–10 correspond to 215, 21.5, 2.15, 0.215 ng, 21.5, 2.15 and 0.215 pg of wheat DNA, respectively.

Table 1

Yield and purity of DNA extracted from thermally processed wheat based food products.

Processing	Samples	DNA yield (ng/mg)	A _{260/280}
Unprocessed	Whole wheat kernel	217 ± 15.8	1.7 ± 0.03
Milling	Whole-wheat flour	230 ± 23.3	1.62 ± 0.04
	Refined flour	169 ± 18.2	1.63 ± 0.03
Extrusion	Vermicelli	159 ± 13.05	1.7 ± 0.03
	Noodles	73 ± 13.45	1.62 ± 0.04
	Flake	35 ± 4.05	1.75 ± 0.04
Deep fat frying	Puri	59 ± 0.29	1.59 ± 0.02
	Samosa	37 ± 5.30	1.53 ± 0.04
Baking	Pizza base	99 ± 5.50	1.67 ± 0.03
	Biscuit	28 ± 3.60	1.68 ± 0.05
	Rusk	27 ± 10.22	1.54 ± 0.08
	Cake	8.0 ± 2.13	1.62 ± 0.06
	Patties	7.2 ± 4.2	1.40 ± 0.05

Results are the mean ± standard deviation. Each sample was analyzed in triplicate.

od would therefore render it suitable for detecting glutenin DNA in thermally processed food products.

2.8. Detection of wheat DNA in thermally processed foods

Several commercial food products were evaluated to detect wheat DNA. The quantity and quality of DNA extracted is governed by the type and degree of thermal processing (Moreano, Busch, & Engel, 2005). The quantity of DNA isolated from processed foods was several orders of magnitude lower than the DNA isolated from unprocessed wheat flour (Table 1). The difficulty in extracting DNA increased in proportion to the severity of processing. Baked products in general yielded less DNA in comparison to either fried or extruded products. The DNA yield decreased with longer thermal exposure time. The 135 bp fragment was efficiently amplified when DNA (75 ng) from whole-wheat flour, refined wheat flour, semolina, chapati, pasta, bread and infant formula were used as template (Fig. 4a). Extracted genomic DNA was evaluated by agarose gel electrophoresis to reveal the extent of degradation. Despite genomic DNA of bread and infant formula, undergoing severe degradation (Fig. 5), the 135 bp fragment was amplifiable, as revealed by agarose gel electrophoresis (Fig. 4a, Lanes 8 and 9). It has been established that shorter DNA (<200 bp) has higher and improved stability. The average size of DNA extracted from bread a highly processed product is ca 300 bp (Fig. 5), yet amplification of the 135 bp fragment was successful. The 135 bp PCR product was gel eluted and subjected to DNA sequencing. The DNA sequence obtained was identical to the wheat DNA sequence 887–1021 (GenBank Accession No. U86029). DNA extracted from highly processed matrices such as cakes and biscuits were amplifiable (Fig. 4b). The amplifiability of the glutenin gene from these samples evinces the suitability of method to thermally processed foods. India is a major producer and consumer of wheat and wheat based products. Therefore thermally processed traditional foods deep-fried, baked and griddle baked were included for detection of wheat by this method. The positive amplification of the 135 bp fragment indicates that this method is applicable to all processed foods tested (Fig. 4b, Lanes 3–12).

In the absence of any available gluten-free labeled foods in the local supermarkets, processed and packaged foods with labels indicating the absence of wheat ingredients were evaluated to validate the method. The absence of the 135 bp PCR product in all the samples tested indicates the absence of any wheat contamination (Fig. 4c). The amplification of 137 bp fragment of 18S rDNA further evinces that the absence of amplification of the glutenin gene is not due to the presence of PCR inhibitors (Fig. 4c).

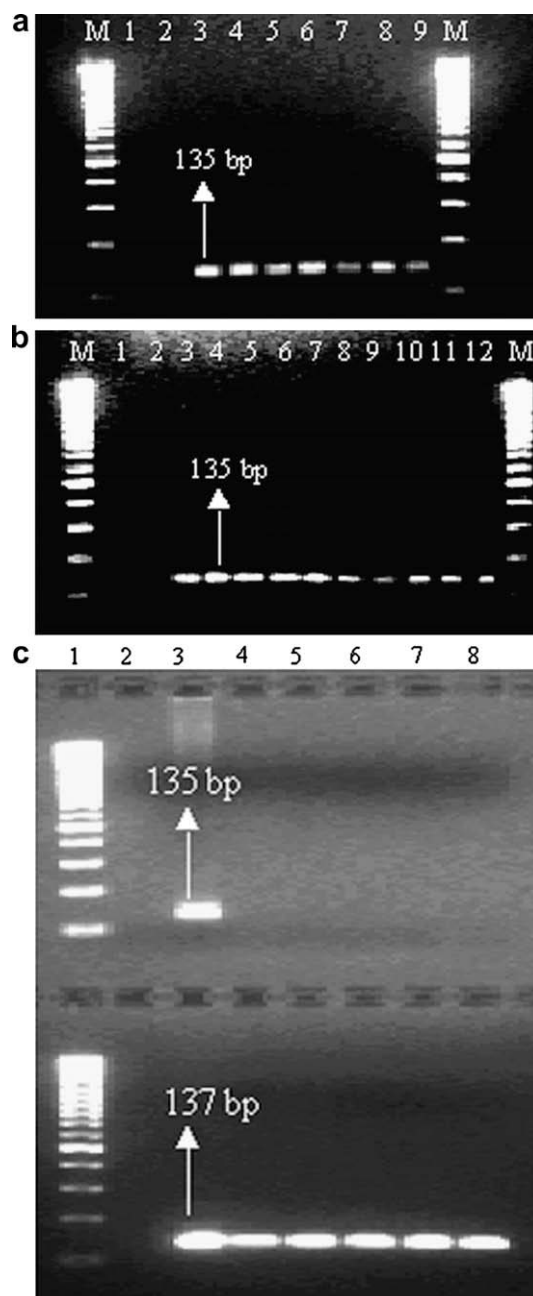


Fig. 4. Agarose gel electrophoresis of PCR products amplified from: (a) Wheat and processed wheat products. Lane M, 100 bp DNA ladder; Lane 1, premise control; Lane 2, negative control; Lane 3, Whole wheat flour; Lane 4, refined wheat flour; Lane 5, semolina; Lane 6, chapati (unleavened bread); Lane 7, pasta; Lane 8, bread; Lane 9, infant formula. (b) Wheat based commercial food samples. Lane M, 100 bp DNA ladder; Lane 1, premise control; Lane 2, negative control; Lane 3, vermicelli; Lane 4, noodles; Lane 5, breakfast cereal; Lane 6, puri; Lane 7, samosa; Lane 8, pizza base; Lane 9, biscuit; Lane 10, rusk; Lane 11, cake and Lane 12, patties. (c) Wheat free commercial food samples. Lane 1, 100 bp DNA ladder; Lane 2, premise control; Lane 3, whole wheat flour; Lane 4, ragi noodles; Lane 5, rice noodles; Lane 6, rice sevai; Lane 7, puffed rice and Lane 8, rice flakes.

In all the above PCR assays, appropriate controls were included. A reaction without template DNA served as premise control. DNA extracted from maize whole flour, which does not contain glutenin served as the negative control (Fig. 4a and b, Lane 2). The absence of amplification products in both the premise and negative control indicate the absence of any contaminating DNA from the environment, buffers or reagents used. The amplification of the 135 bp

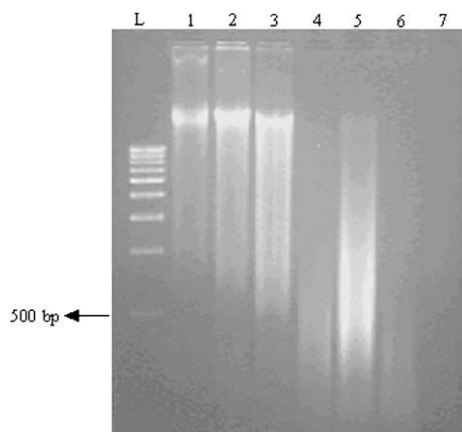


Fig. 5. Agarose gel electrophoresis showing degraded genomic DNA extracted from heat processed foods. Lane M, 500 bp DNA ladder; Lane 1, whole wheat flour; Lane 2, refined wheat flour; Lane 3, semolina; Lane 4, chapati (Unleavened bread); Lane 5, pasta; Lane 6, Bread; Lane 7, infant formula.

fragment of glutenin required 75 ng of template DNA which was also sufficient for highly processed samples. This can be explained by the high copy number (35–40) of the low molecular weight glutenin gene as determined by Southern blot analysis (Cassidy, Dvorak, & Anderson, 1998). Further, the target sequence of <200 bp facilitates detection of highly fragmented DNA. Commercially gluten-free products contain rice, maize or soya based and wheat contamination is possible at every stage right from farm to finished table products. Therefore both the raw ingredients as well as finished foods need to be certified as gluten-free. Commercial immunochemical methods for the detection of gluten proteins are available with a lower limit of 10 ppm wheat gluten. However these tests exhibit cross reactivity to both barley and rye gluteins (Lardizabal et al., 2002). Heat denaturation of gluten proteins in thermally processed products can yield false negative results (Booth, Losowsky, Walkersmith, & Whitney, 1991). Allmann, Candrian, and Lüthy (1992) amplified a 159 bp fragment of the gibberlin responsive gene of wheat from wheat bread, this however is not suitable for labeling gluten-free foods. They conclude that to obtain a positive signal the average size of amplicon must be >159 bp. In contrast the method presented here indicates that it is possible to detect a smaller target amplicon size of 135 bp. The method described targeting the low molecular weight glutenin gene can therefore be used to detect wheat in raw ingredients (flours) all the way to the finished product. Thus ensuring food safety for people who need to avoid the consumption of wheat and products derived there from. Though previous studies have reported PCR based assays for certifying gluten-free foods (Allmann et al., 1992; Dahinden et al., 2001; Olexova et al., 2006; Sandberg et al., 2003) most of these assays do not distinguish between wheat, barley and rye. The PCR method described in the present study unequivocally identifies a wheat ingredient in a plethora of processed foods. The qualitative detection of wheat in spiked flours containing 0.1% wheat as well as a number of heat processed products makes this DNA based detection complementary to immunochemical methods in the in vitro safety and labeling of foods for the wheat allergics and celiac sprue patients

3. Conclusions

A major impediment in the adherence of gluten-free diet is that gluten is often a hidden ingredient in foods. All foods require clear identification on labels to enable wheat allergy and CD patients to make informed choices. Dieticians and clinicians have a responsi-

bility to help their patients identify hidden sources of wheat and gluten. This advocates the need for newer analytical tools for identifying gluten in food products. Wheat, other than rye and barley is the most potential gluten contaminant. The PCR method described targeting the LMW glutenin gene is very specific for wheat and can be efficiently used to detect the presence of wheat in food. The method can be applied to heat treated/processed food products. The small amplicon size (135 bp) is in part responsible for the successful outcome of these analyses. Moreover the sensitivity of this method contributes to the adequacy of these methods for compliance with Codex Alimentarius. The extracted DNA of wheat in food samples expected to be fragmented as a consequence of multiple processing parameters is still detectable. This method supplements the existing immunochemical methods for testing gluten free food. Greater cooperation and collaboration is also required between the food manufacturers, food service industry, regulatory agencies, clinicians and dieticians to facilitate adherence to gluten-free foods by using current state of art techniques.

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