Validation of Molecular Detection Methods for Gluten Allergens in Infant Formula

Shuhuan ZHAO, Yunxia WANG, Cuizhi LI*

Inner Mongolia Yili Industrial Group Co., Ltd., Inner Mongolia, Hohhot 010110, China

Abstract [**Objectives**] To verify the specificity, sensitivity, precision and negative-positive deviation of the foodproof gluten component detection kit for the detection of gluten allergens in milk powder matrix, and to establish a real-time fluorescent PCR legal method for the detection of gluten allergens in milk powder. [**Methods**] The specificity, sensitivity, precision and negative-positive deviation of the detection method of foodproof gluten component detection kit (PCR-probe method) were verified by artificially adding different concentrations of wheat bran and extracting sample DNA by kit method, and applied to sample detection. [**Results**] The specific detection results of two kinds of milk powder with wheat bran and buckwheat added showed that the foodproof gluten component detection kit (PCR-probe method) had good specificity for wheat gluten. The results of artificially added wheat bran positive samples showed that the false positive rate and false negative rate of the kit in the milk powder matrix were 0, and the sensitivity and precision were high. [**Conclusions**] The kit is simple to operate and has high accuracy, which is suitable for the detection of gluten allergen components in milk powder.

Key words Real-time fluorescent PCR, Gluten, Allergen, Infant formula

1 Introduction

Food allergy is a recurrent, adverse reaction caused by a specific immune response after exposure to a particular food and has become a global food safety issue[1-2]. The presence of natural allergenic components in food ingredients causes allergic reactions in 5% - 8% of children and 2% - 4% of adults, which can be severe and life-threatening[3]. Among them, gluten is one of the food allergen components and was the first food allergen to receive international attention and research. Gluten is a structural protein complex widely found in wheat grains, especially barley, wheat, oats and other grains, mainly composed of wheat alcohol soluble proteins and wheat gluten proteins, which account for 80% - 85% of the total protein content of wheat[4-6]. Gluten proteins are the common antigens of celiac disease and immunoglobulin E (IgE)-mediated wheat allergy, and when an allergic individual is exposed to this protein for the first time, the body's immune system produces antibodies that become IgE, and upon re-exposure to the same food proteins, the IgE antibodies and chemicals such as histamine are released, causing respiratory, gastrointestinal, dermal, or cardiovascular reactions that, in severe cases may even be fatal[7-8].

The techniques used for allergen detection can be categorized into two main groups, *i. e.*, immunological detection techniques based on the protein level, and molecular biology detection techniques based on the gene level[9]. Molecular biology detection techniques based on the gene level include real-time fluorescence quantitative PCR, multiplex PCR, reverse transcription PCR, digital PCR, *etc.* Immunological detection techniques based on the protein level include electrophoresis, mass spectrometry, immunological methods and enzyme-linked immunosorbent assay. The most widely used are mainly ELISA and PCR methods[10-11]. Since the accuracy and specificity of immunological detection methods mainly depend on the recognition of allergens by antibodies, certain food industries often utilize deep-processing techniques such as thermal processing, ultra-high pressure and enzymatic digestion to deform and degrade allergenic proteins, and changes in protein structure can interfere with protein extracts or antibody binding sites, which can also lead to the emergence of false-negative results[12]. Real-time fluorescence PCR method, as a new detection technology, has played an increasingly important role in allergen detection, and it effectively solves the problems of immunological methods, such as the inability to detect processed foods and high false-negative rate, and the instrument is relatively inexpensive, with low requirements for personnel experience and operation, and the detection cost is extremely low, high accuracy, good reproducibility, applicability, and easy to popularize the application[13]. The foodproof Gluten Composition Detection Kit (PCR-Probe Method) adopts real-time fluorescent PCR technology to design primers and probes for wheat- and barley-specific genes, and the probe bound to the template is broken down by Taq enzyme to produce fluorescent signals during PCR amplification, and the fluorescent quantitative PCR instrument plots real-time amplification curves based on the detected fluorescent signals, thus realizing the qualitative detection of gluten at the nucleic acid level. The qualitative detection of gluten at the nucleic acid level is realized.

In this study, the specificity, sensitivity and precision of the

Received: November 10, 2023 Accepted: January 5, 2024

Shuhuan ZHAO, master, intermediate engineer, research fields: food engineering.

Yunxia WANG, bachelor, engineer, research fields: animal medicine.

* Corresponding author. Cuizhi LI, master, professor-level senior engineer, research fields: food safety prevention and control.

foodproof gluten detection kit were verified, and the false-positive and false-negative rates of the kit were also evaluated by milk powder samples artificially spiked with wheat gluten, with a view to providing more choices of detection protocols for the detection of gluten allergenic components in milk powder.

2 Materials and methods

2.1 Samples and reagents

2.1.1 Sample information. Infant formula, wheat bran, buckwheat, samples from Jingdong e-commerce and a market in Hohhot City.

2.1.2 Reagents. foodproof gluten component detection kit (BIOTECON Diagnostics Company), plant gene extraction kit (Tiangen Biochemical Technology Co., Ltd.).

2.2 Instruments and equipment Pico17 benchtop centrifuge (Thermo-Fisher, USA), SLAN-96P real-time fluorescence quantitative PCR instrument (Shanghai Hongshi Medical Technology Co., Ltd.), A2 type biological safety cabinet (Thermo-Fisher, USA), BioDrop ulite micro nucleic acid protein assay (Bio-Drop, USA).

2.3 Experimental methods

2.3.1 Extraction of template DNA. Weighed 30 mg of each infant formula sample and simulated gluten-containing sample in a 2 mL centrifuge tube, extracted and purified the template DNA using the DNA extraction and purification kit, and then used the micronucleic acid protein assay to determine the quality and concentration (OD_{260}/OD_{280} is in the range of 1.7 – 2.0) for fluorescence PCR detection.

2.3.2 Foodproof gluten allergen component detection kit methods. First, the solution was thawed. To maximize recovery of the contents, shook the vial briefly in a microcentrifuge before opening. Removed the assay master mix, blew up and down to mix carefully, pipetted 20 μL of PCR Master Mix into each PCR tube, added 5 μL each of the negative control, sample DNA extract, and positive control into PCR tubes, covered the tube, centrifuged briefly, and immediately performed the PCR amplification reaction. The PCR tubes were placed on a PCR instrument, and the recommended reaction program was set as follows: reaction system 25 μL. The recommended reaction program is as follows: 25 μL of reaction system, fluorescence signal is detected at 60 ℃ in each cycle of the second step, and the detection channel is selected as FAM, and the detailed operation procedure is described in the instruction manual.

2.3.3 Specificity verification. Took infant formula as the base, separately added 1% wheat gluten and buckwheat, extracted the DNA of the samples for specificity test according to the method described in the DNA extraction kit, and carried out the PCR reaction and interpretation of the results according to the method in Section **2.3.2** in the instructions of Foodproof Gluten Allergen Component Detection Kit to verify the specificity of the method.

2.3.4 Sensitivity validation. Wheat gluten and infant formula were mixed in different proportions to obtain samples with different gluten contents, and the gluten contents were 100%, 10%, 1%, 0.1%, 0.01%, and 0.001%, with a total of 6 concentration gradient samples, and the DNA of the samples used for sensitivity testing was extracted in accordance with the method described in the DNA Extraction Kit, and the DNA of the samples was extracted in accordance with the method in Section **2.3.2**, and PCR reaction and result interpretation were performed according to the method in Section **2.3.2** to verify the sensitivity of the method.

2.3.4 Precision verification. Took 2 samples of infant formula and infant formula with artificially added 0.1% wheat gluten, extracted the DNA of the samples used for the precision test according to the method described in the DNA extraction kit, and carry out PCR reaction 10 times for each sample according to the setting of the instruction manual of Section **2.3.2** Foodproof Gluten Allergen Ingredient Detection Kit to validate the precision of the method.

2.3.5 Negative and positive bias experiment verification. Took 5 samples of infant formula, 20 samples of infant formula with 0.01% wheat gluten added artificially, and 5 samples of infant formula with 0.1% wheat gluten, extracted the DNA of the samples used in the negative-positive deviation experiment according to the method described in the DNA extraction kit, and carry out the PCR reaction and interpretation of the results in accordance with the setting of the instruction manual of Section **2.3.2** Foodproof Gluten Allergen Ingredient Detection Kit. PCR reaction and result interpretation were performed to calculate the false positive rate and false negative rate to verify the negative and positive deviation of the method.

3 Results and analysis

3.1 Specificity test results The *Ct* value of foodproof Gluten Allergen Detection Kit for infant formula containing 1% wheat gluten was less than 35, and the result was determined as "positive for gluten-containing cereal"; while no specificity was found for infant formula containing 1% buckwheat and infant formula without wheat gluten. The *Ct* value was No *Ct*, and the result was negative for gluten-containing cereal ingredients. The results are shown in Table 1, which indicates that the specificity of the kit is good.

Table 1 Test results of specificity for Foodproof Gluten Allergen ingredient test kit

No.	Sample Name	PCR Result
1	Infant formula	– (No *Ct*)
		– (No *Ct*)
		– (No *Ct*)
2	Infant formula with artificially added 1% wheat bran	+ (24.32)
		+ (23.77)
		+ (24.08)
3	Infant formula with artificially added 1% buckwheat	– (No *Ct*)
		– (No *Ct*)
		– (No *Ct*)

3.2 Sensitivity test results The results of the sensitivity test

are shown in Table 2. From the results, it can be concluded that the content of wheat gluten can still be detected at 0.01%, which indicates that the sensitivity of this method can reach 0.01%, and that it can also detect wheat gluten at 0.01%. The sensitivity of this method can reach 0.01%.

3.3 Results of precision experiment Using infant formula as the substrate, one negative sample without wheat gluten, one positive sample with artificial contamination of 0.1% wheat gluten, each sample was repeated 10 times. The results of the precision test are shown in Table 3. From the results, it can be concluded that the results of the precision experiment of the foodproof gluten allergen component detection kit are consistent.

3.4 Results of negative and positive deviation experiments The results of the negative-positive deviation test are shown in Table 4. The results of the negative-positive deviation test are calculated from the results in Table 4. The false-positive rate of the results was 0. For positive results, the false-negative rate of the foodproof Gluten Allergen Ingredient Detection Kit test results was 0.

Table 2 Test results of sensitivity for Foodproof Gluten Allergen ingredient test kit

No.	Pollution concentration//%	PCR results		
1	100.0	+(19.82)	+(20.04)	+(18.96)
2	10.0	+(20.14)	+(18.52)	+(20.13)
3	1.0	+(22.65)	+(23.00)	+(22.89)
4	0.1	+(21.38)	+(23.84)	+(24.93)
5	0.01	+(24.54)	+(23.22)	+(19.17)
6	0.001	-(No *Ct*)	-(No *Ct*)	-(No *Ct*)

Table 3 Test results of precision for Foodproof Gluten Allergen ingredient test kit

No.	Sample name	PCR result				
1	Infant formula with artificially added 0.1% wheat bran	+(24.35)	+(19.86)	+(22.37)	+(23.38)	+(24.22)
		+(25.17)	+(24.33)	+(24.32)	+(21.64)	+(23.51)
2	Infant formula	-(No *Ct*)	-(No *Ct*)	-(No *Ct*)	-(No *Ct*)	-(No *Ct*)
		-(No *Ct*)	-(No *Ct*)	-(No *Ct*)	-(No *Ct*)	-(No *Ct*)

Table 4 Test results of negative-positive deviation for Foodproof Gluten Allergen ingredient test kit

Sample name	Samples/pcs	Samples positive/pcs	Samples negative/pcs	Positive/Negative deviation results
Negative samples	5	0	5	0
Positive sample (0.01%)	20	20	0	0
Positive sample (0.1%)	5	5	0	0

4 Conclusions and discussion

Abnormal or abnormal immune responses in the human body caused by food allergy have become a global public health and food safety issue of great concern[14]. With the rapid development of molecular technology at the DNA level, high-throughput and highly sensitive PCR technology has also played an important role in allergen detection, and the use of real-time fluorescent PCR to detect gluten allergen components is easier in the pre-treatment process and more accurate in the results compared with mass spectrometry methods, immunological methods, enzyme-linked immunosorbent assay, *etc*[15-18].

This test was conducted to validate the specificity, sensitivity, precision and negative and positive deviations of the fluorescence quantitative PCR assay by measuring the mixed samples of adulterated wheat gluten. From the test results, it can be seen that the gluten components in milk powder can be detected quickly and accurately by real-time fluorescence quantitative PCR, and the foodproof gluten allergen component detection kit has good specificity, sensitivity, precision, and the results of the negative and positive deviations were all 0. The kit has the advantages of simple operation and rapidity, which can meet the needs of the rapid detection of infant milk powder samples, and it is suitable for monitoring the gluten allergen composition of milk powder used in the production and processing process, providing powerful technical support for the quality control of enterprise products.

References

[1] RENZ H, ALLEN K J, SICHERER S H, *et al.* Food allergy[J]. Nature Reviews Disease Primers, 2018(4): 17098.

[2] CHEN BH, WU YH, WU H, *et al.* Progress of the association between food allergy and inflammatory bowel disease[J]. Food Science, 2023, 44(1): 294-304. (in Chinese).

[3] BOGH KL, MADSEN CB. Food allergens: Is there a correlation between stability to digestion and allergenicity[J]. Critical Reviews in Food Science and Nutrition, 2016, 56(9/12): 1545-1567.

[4] FU LL, WANG YB. Food allergy: From sensitization mechanisms to control strategies[J]. Food Science, 2021, 42(19): 1-19. (in Chinese).

[5] ZHANG S, HAN B, REN LK. Current status of research on quality enhancement of gluten-free foods[J]. Journal of Food Safety and Quality Testing, 2022.8,13(15): 4899-4907. (in Chinese).

[6] CABANILLAS B. Gluten-related disorders: Celiac disease, wheat allergy, and nonceliac gluten sensitivity[J]. Critical Reviews in Food Science and Nutrition, 2019, (5): 1-16.

[7] HU XJ. Digestive stability and potential sensitization of gluten proteins in seventeen wheat germplasm species[D]. Nanchang: Nanchang University, 2021. (in Chinese).

[8] YANG L, WANG Z. Determination of gluten allergens by ELISA and evaluation of its uncertainty[J]. Modern Foods, 2022: 182-186. (in Chinese).

(To page 36)

being used as cosmetic raw materials, natto skin care soap, health food and medicine, and have a broader development prospect.

References

[1] LI KX, LI SB, YANG WQ, *et al.* Research progress of *Bacillus natto* [J]. Genomics and Applied Biology, 2021,40(Z3): 3131 - 3139. (in Chinese).

[2] LIU HH, MIAO XY, ZHENG L, *et al.* Changes in nutrient contents and active substances during natto production and cold storage[J]. Food Research and Development, 2021,42(4): 48 - 54. (in Chinese).

[3] YAO MJ, YANG Y, FAN J, *et al.* Advance on nattokinase microbial production and physiological function[J]. Science and Technology of Food Industry, 2022,43(14): 435 - 444. (in Chinese).

[4] FUKUTAKE M. Quantification of genistein and genistinin beans and soybean products[J]. Food Chemical Toxicology, 1996(34): 457 - 461.

[5] CHU JX, ZHAO HZ, LU ZX, *et al.* Improved physicochemical and functional properties of dietary fiber from millet bran fermented by *Bacillus natto*[J]. Food Chemistry, 2019, 294(10): 79 - 86.

[6] SUMI H. Accumulation of vitamin k(menaquinone-7) in plasma after ingestion of natto and natto bacilli(*B. subtilis* natto)[J]. Food Science and Technology Research, 1999, 5(1): 48 - 50.

[7] WANG SX, REN YJ, YUAN N. Research progress on natto and its functional properties[J]. Cereals & Oils, 2020, 33(6): 21 - 23. (in Chinese).

[8] NIAN A. Natto: A prevention expert of cardiovascular and cerebrovascular diseases[J]. China Food Industry, 2021(11): 102. (in Chinese).

[9] YAN QX, FENG L, XU F, *et al.* Thrombolytic effect of nattokinase and its mechanism[J]. Science and Technology of Food Industry, 2021, 42(24): 340 - 346. (in Chinese).

[10] SONG JX, QI HB, SU XY. Antibacterial activity of *Bacillus natto*[J]. Soybean Science, 2022,41(2): 203 - 208. (in Chinese).

[11] CHANG XH, HUANG W, HAN YD, *et al.* Study on high antimicrobial activity induction techniques of *Bacillus natto*[J]. Heilongjiang Animal Science and Veterinary Medicine, 2019(9): 99 - 103. (in Chinese).

[12] YU XJ, LI YT, LIU R, *et al.* The preparation of antiseptic seafood seasoning by fermentation of tuna scraps[J]. The Food Industry, 2021, 42(4): 62 - 66. (in Chinese).

[13] CAI TQ, LI Y, XU JW, *et al.* Study on the effect of natto extract on the prevention of osteoporosis[J]. Chinese Journal of Osteoporosis, 2022, 28(1): 26 - 30. (in Chinese).

[14] QI HB, SONG JX, LIN HP, *et al.* Adjusting to the intestinal microflora of fermented bean dregs extract by *Bacillus natto*[J]. The Food Industry, 2019, 40(2): 5 - 8. (in Chinese).

[15] HATAKEYAMA SHIORI, KAFUKU MAYU, OKAMOTO TOMOYA, *et al.* Studies on the anticancer mechanisms of the natto extract[J]. Journal of Society of Materials Engineering for Resources of Japan, 2016, 27(1 - 2): 15 - 19.

[16] CORTÉS VIGURI, VIANNEY, HERNÁNDEZ RODRÍGUEZ, *et al.* Annatto (*Bixa orellana* L.), a potential novel starch source: antioxidant, microstructural, functional, and digestibility properties[J]. Journal of Food Measurement and Characterization, 2021, (11): 1 - 15.

[17] TAMURA MOTOI, WATANABE JUN, HORI SACHIKO, *et al.* Effects of a high-γ-polyglutamic acid containing natto diet on liver lipids and cecal microbiota of adult female mice[J]. Bioscience of Microbiota, Food and Health, 2021, 40(4):176 - 185.

[18] SUN RY, LI D, SUN MB, *et al.* *Bacillus natto* ameliorates obesity by regulating PI3K/AKT pathways in rats[J]. Biochemical and Biophysical Research Communications, 2022(603): 160 - 166.

[19] QI HB, LU P, CHEN J. The method of detecting hydroxyl radical scavenged by faded safranine[J]. Journal of Xinyang Normal University (Natural Science Edition),2009(3): 448 - 450. (in Chinese).

[20] XIE DD, ZHANG L, SUN YQ. Comparative study on fermentation and antioxidant properties of different kinds of fruit enzymes[J]. Journal of Henan University of Technology (Natural Science Edition), 2022, 43(1): 35 - 41. (in Chinese).

(From page 31)

[9] NING YW, YANG Z, MA MG, *et al.* Research progress on common allergens in food and detection techniques[J]. Food Science, 2021, 42(15): 319 - 328. (in Chinese).

[10] FENG YJ, YUAN JL, TONG P, *et al.* Progress of detection methods for soybean allergens[J]. Food Science, 2012, 33(23): 365 - 369. (in Chinese).

[11] XU YY, SU SL, XU PQ, *et al.* Determination of gluten allergens in cereal supplements for infants and young children by real-time fluorescence PCR method[J]. Journal of Food Safety and Quality, 2019.4, 10(8): 2350 - 2355.

[12] CHEN Y, WANG W. Research progress on food allergen detection methods[J]. Journal of Quarantine and Inspection, 2011, 21(3): 4 - 7.

[13] SHANG K, ZHANG B, DUAN QZ, *et al.* Real-time fluorescence PCR for the detection of allergenic components in wheat and hazelnut[J]. Food and Fermentation Industry, 2018, 44(4): 234 - 240.

[14] ZENG XQ, CHEN YY, HAN ZJ, *et al.* Progress of multiple nucleic acid detection technology for plant-based allergens[J]. Modern Foods, 2022, 28(22): 43 - 46. (in Chinese).

[15] ZHU XY, YUAN JL, CHEN HB, *et al.* Detection methods for wheat gluten related to celiac disease: A review[J]. Science and Technology of Food Industry, 2012, 33(23): 419 - 423. (in Chinese).

[16] KE YN, GE Y, CHAO QG, *et al.* Establishment of a routine PCR method for the detection of wheat sensitizing components in foodstuffs [J]. Food and Fermentation Industry, 2011, 37(7): 184 - 187. (in Chinese).

[17] PAFUNDO S, GULLI M, MARMIROLI N. Multiplex real-time PCR using SYBR GreenER for the detection of DNA allergens in food[J]. Analytical and Bioanalytical Chemistry, 2010, 396(5): 1831 - 1839. (in Chinese).

[18] WANG W, HAN JX, WU YJ, *et al.* Multiplex PCR for the detection of eight food allergens including wasabi[J]. Food and Fermentation Industry, 2011, 37(6): 156 - 160. (in Chinese).