- Preparation of samples
  ↓
- Polyacrylamide gel electrophoresis (PAGE)
  ↓
- Blotting
  ↓
- Blocking (TBS-T with 0.1% BSA)
- Reaction with 1st antibody
- Reaction with 2nd antibody
- Reaction with avidin-labeled alkaline phosphatase (AP)–biotin conjugate
- AP reaction with substrate
  ↓
- Detection of protein-derived allergens

FIGURE 4.4 Flowchart of procedures for Western blotting.

3. PCR method for wheat, buckwheat, peanut, shrimp, and crab

PCR is a DNA-based method that is very specific and sensitive for detection of specific allergenic ingredients in processed foods. The PCR method was established as the confirmation test for wheat, buckwheat, and peanut in the Japanese official methods. Three DNA extraction methods (silica-membrane column-type kit, anion-exchange column-type kit, and CTAB method) are prescribed in the Japanese official methods. The PCR target genes for detection of wheat (Yamakawa *et al.*, 2007a), buckwheat (Yamakawa *et al.*, 2008), and peanut (Watanabe *et al.*, 2006) are shown in Table 4.10. The primer pairs were designed to detect these gene sequences. To check the validity of the extracted DNA for PCR quality, primers recognizing the noncoding region of the chloroplast DNA were designed as the analytical control (Watanabe *et al.*, 2006). To avoid a false-negative result, it is important to check the validity of the extracted DNA for PCR.

Since the MHLW designated shrimp/prawn and crab for mandatory labeling in June 2008, respective PCR methods to discriminate between shrimp/prawn and crabs in processed foods have been developed.

Both methods have been validated according to the Japanese validation protocol (Sakai *et al.*, 2008), and both primers are commercially available. All the Western blotting and PCR kits are shown in Table 4.11.

E. Validation study

We performed collaborative studies using the ELISA methods with model processed foods (sausage, boiled beef in an aluminum pouch, tomato sauce, biscuit, juice, and jam) containing allergen proteins. The six

TABLE 4.10 PCR for wheat, buckwheat, peanut, shrimp and crab

Methods for DNA extraction	
Silica-membrane column-type kit	
Qiagen DNeasy Plant Mini kit	
Anion-exchange column-type kit	
Qiagen Genomic-tip kit	
CTAB method	
PCR target gene sequences	
Wheat	Triticin precursor gene
Buckwheat	Gene encoding soba allergenic protein
Peanut	Agglutinin precursor gene
Shimp/prawn	16S rRNA gene of mitochondrial DNA
Crab	16S rRNA gene of mitochondrial DNA
Plant	Noncoding region of chloroplast DNA
Animal	16S rRNA gene of mitochondrial DNA

model processed foodstuffs were spiked with specific allergenic ingredients to final levels of 10 μg/g in the ingredient stage (Matsuda *et al.*, 2006; Sakai *et al.*, 2008). We considered that using the model processed foods would be the best way to assess the established ELISA methods by interlaboratory validation. First of all, we conducted a homogeneity test for the model processed foods. Basically, the procedure was performed following the AOAC homogeneity test protocol with some modifications, as described in Table 4.12. The sausage, boiled beef in an aluminum pouch, and tomato sauce were evaluated using a Nippon Meat Packer kit. The biscuit, orange juice, and jam were evaluated using the Morinaga kit.

Table 4.13 shows the method for interlaboratory validation. The first step is the preparation of a standard curve (4-parameter logistic curve) using the absorbance value collected from each participating laboratory. Second, the first and second sets of data are subjected to repeatability using the average values from three wells. Third, Cochran's test and Grubbs's test are used for the removal of outliers (both tests were performed at a significance level of 5%). The final step was estimation of one-way analysis of variance (ANOVA). The 10 participating laboratories included manufacturing companies, public research institutes, local public inspection institutes, and private inspection institutes. Tables 4.14–4.19 show the validation results for egg, milk, wheat, buckwheat, peanut, and shrimp/prawn (crustacean), respectively (Matsuda *et al.*, 2006; Sakai *et al.*, 2008).

These results were evaluated according to the AOAC protocol and ISO 5725-5 robust statistics. Both kits meet the Japanese acceptance criteria.

TABLE 4.11 Commercial Western blot and PCR kits for specific allergenic ingredients

Specific allergenic ingredient	Western blot or PCR kits	Target protein or gene	Relative molecular weight (Da) or PCR product length (bp)
Egg	Morinaga FASPEK Egg Western Blot Kit (ovalbumin) Morinaga Institute of Biological Sciences Co.	Ovalbumin	50,000
	Morinaga FASPEK Egg Western Blot Kit (ovomucoid) Morinaga Institute of Biological Sciences Co.	Ovomucoid	38,000
Milk	Morinaga FASPEK Milk Western Blot Kit (β-lactoglobulin) Morinaga Institute of Biological Sciences Co.	β-lactoglobulin	18,400
	Morinaga FASPEK Milk Western Blot Kit (Casein) Morinaga Institute of Biological Sciences Co.	Casein	33,000–35,000
Wheat	Allergen checker [Wheat] Oriental Yeast Co., Ltd.	Triticin precursor gene	141
Buckwheat	Allergen checker [Buckwheat] Oriental Yeast Co., Ltd.	Gene encoding soba allergenic protein	127
Peanut	Allergen checker [Peanut] Oriental Yeast Co., Ltd.	Agglutinin precursor gene	95
Shrimp	Primer for shrimp detection, FASMAC Co., Ltd.	16S rRNA gene of mitochondrial DNA	187
Crab	Primer for crab detection, FASMAC Co., Ltd.	16S rRNA gene of mitochondrial DNA	62
Plant DNA	Allergen checker [Plant] Oriental Yeast Co., Ltd.	Noncoding region of chloroplast DNA	Approximately 124
Animal DNA	Allergen checker [Animal] Oriental Yeast Co., Ltd.	16S rRNA gene of mitochondrial DNA	370–470

TABLE 4.12 Homogeneity test for model processed foods[a]

1. Randomly select 3 g × 6 samples (n).
2. Take 1 g × 2 test portions (p) from each 3-g sample.
3. Analyze the $2n$ test portion ($12p$) in random order under repeatable conditions (two wells).
4. Estimate the sampling variance (S^2s) by one-way analysis of variance ($2 \times 6n$) using the average value of each well (estimation variance between each portion and each sample).
5. Estimate the analytical variance (S^2a) by one-way analysis of variance ($2 \times 12p$) using each well value (estimation variance between each well and each portion).

[a] The procedure basically follows the AOAC homogeneity test protocol with some modification.

TABLE 4.13 Evaluation method for the interlaboratory study

Step 1
The standard (four-parameter logistic) curve was prepared by the simplex method using absorbance values collected from each participating laboratory.

Step 2
The first and second portion data were subjected to a repeatability test using the average values from three wells.

Step 3
Cochran test and *Grubbs test* were performed for the removal of outliers with a significance level of 5%.

Step 4
Estimation of the analytical variance by one-way ANOVA (2 portions × 10 laboratories).

F. Practical test for monitoring the allergy-labeling system.

Figure 4.5 shows the outline of the practical test for monitoring the allergy-labeling system at a local government inspection center. First, we investigated food allergy labeling. As a screening test, quantitative analyses using two different ELISA kits for specific allergenic ingredients were performed to double-check each allergen. We determined the threshold for a positive value to be 10 μg/g in the screening test according to the definition of trace amounts described in Section III.A. Next, we

TABLE 4.14 Recovery, repeatability, and reproducibility for egg detection[a]

Sample	Number of labs	Recovery (%)	Repeatability (%)	Reproducibility (%)
FASTKIT ELISA Ver. II				
Rice gruel	13	85.1	4.3	9.4
Sweet adzuki-bean soup	13	96.0	3.4	9.2
Steamed fish paste	13	83.7	3.6	9.0
Meatball	13	86.1	3.7	8.8
Coffee jelly	13	98.3	3.1	8.5
Fermented soybean soup	13	88.7	3.1	8.8
FASPEK ELISA				
Sausage	10	70.3	4.8	17.4
Boiled beef	9	76.0	3.7	8.0
Cookie	9	51.5	3.5	10.8
Orange juice	10	81.2	3.6	14.2
Jam	9	86.7	4.8	9.4

[a] To confirm the validity of DNA extracted from plants for the PCR and for specific detection of egg.

TABLE 4.15 Recovery, repeatability, and reproducibility for milk detection[a]

Sample	Number of labs	Recovery (%)	Repeatability (%)	Reproducibility (%)
FASTKIT ELISA Ver. II				
Rice gruel	12	89.2	3.4	4.4
Sweet adzuki-bean soup	12	100.3	3.4	5.6
Steamed fish paste	11	74.4	3.7	4.0
Meatball	13	80.8	3.2	8.3
Coffee jelly	12	96.7	4.1	4.5
Fermented soybean soup	13	73.6	4.0	9.9
FASPEK ELISA				
Sausage	10	109.3	5.4	14.9
Boiled beef	10	115.1	7.1	12.2
Cookie	10	96.8	4.9	17.4
Orange juice	10	89.2	4.7	13.3
Jam	10	137.0	2.7	12.1

[a] To confirm the validity of DNA extracted from plants for the PCR and for specific detection of milk.

TABLE 4.16 Recovery, repeatability, and reproducibility for wheat detection[a]

Sample	Number of labs	Recovery (%)	Repeatability (%)	Reproducibility (%)
FASTKIT ELISA Ver. II				
Rice gruel	13	138.9	4.5	9.0
Sweet adzuki-bean soup	13	126.9	3.4	9.9
Steamed fish paste	11	124.4	4.2	5.3
Meatball	13	111.4	5.0	9.0
Coffee jelly	13	129.0	5.1	9.2
Fermented soybean soup	13	110.5	5.7	10.4
FASPEK ELISA				
Chicken meatball	10	92.2	6.2	16.2
Steamed fish paste	10	115.0	10.9	12.9
Orange juice	10	111.7	5.4	11.7
Pudding	10	129.6	6.4	10.6
Mixed stew	10	128.3	6.7	12.0
Tomato sauce	10	122.4	7.0	10.2

[a] To confirm the validity of DNA extracted from plants for the PCR and for specific detection of buckwheat.

TABLE 4.17 Recovery, repeatability, and reproducibility for buckwheat detection[a]

Sample	Number of labs	Recovery (%)	Repeatability (%)	Reproducibility (%)
FASTKIT ELISA Ver. II				
Rice gruel	13	117.5	5.8	18.0
Sweet adzuki-bean soup	13	137.2	6.7	13.3
Steamed fish paste	13	123.0	3.5	10.0
Meatball	13	91.1	7.8	12.7
Coffee jelly	13	112.2	6.6	10.8
Fermented soybean soup	13	93.8	5.4	12.9
FASPEK ELISA				
Sausage	9	101.1	4.5	7.6
Boiled beef	10	121.8	6.5	20.2
Tomato sauce	10	146.1	14.5	17.6
Cookie	10	149.1	8.4	13.4
Orange juice	10	145.7	12.3	17.2

[a] To confirm the validity of DNA extracted from plants for the PCR and for specific detection of buckwheat.

TABLE 4.18 Recovery, repeatability, and reproducibility for peanut detection[a]

Sample	Number of labs	Recovery (%)	Repeatability (%)	Reproducibility (%)
FASTKIT ELISA Ver. II				
Rice gruel	13	74.9	2.5	7.9
Sweet adzuki-bean soup	12	88.9	3.4	7.3
Steamed fish paste	13	100.5	2.5	12.9
Meatball	13	104.1	3.2	12.6
Coffee jelly	13	75.6	3.5	9.7
Fermented soybean soup	13	52.1	2.8	7.8
FASPEK ELISA				
Fermented soybean soup	12	86.8	2.8	4.8
Chicken meat ball	11	87.5	2.0	4.9
Jerry	12	89.1	4.2	5.5
Orange juice	11	84.6	3.0	5.7
Corn soup	12	104.7	2.4	5.7
Tomato sauce	12	109.6	3.5	6.2

[a] To confirm the validity of DNA extracted from plants for the PCR and for specific detection of peanuts.

TABLE 4.19 Recovery, repeatability, and reproducibility for shrimp/prawn detection[a]

Sample	Number of labs	Recovery (%)	Repeatability (%)	Reproducibility (%)
Crustacean kit [Maruha]				
Fish sausage	10	102.8	5.1	23.2
Freeze-dried egg soup	9	98.3	4.1	19.4
Tomato sauce	10	95.8	9.7	19.7
Creamy croquette	10	82.1	8.2	20.6
Chicken ball	10	100.0	6.6	21.6
FA test EIA–Crustacean [Nissui]				
Fish sausage	8	63.5	4.0	6.1
Freeze-dried egg soup	10	73.6	3.9	9.4
Tomato sauce	10	85.7	4.6	5.5
Creamy croquette	10	77.7	4.8	6.2
Chicken ball	10	72.2	5.1	8.9

[a] To confirm the validity of DNA extracted from plants for PCR and for specific detection of shrimp/prawn.

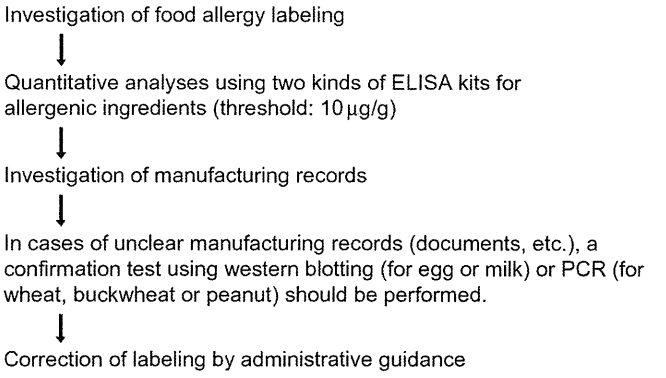

FIGURE 4.5 Outline of the practical test used to monitor the allergy-labeling system.

examined the manufacturing records. If the presence of an allergen cannot be elucidated, a confirmation test using Western blotting for egg or milk or PCR for wheat, buckwheat, peanut, shrimp/prawn, or crab should be performed. If an allergen can be positively detected using the confirmation test, labeling should be corrected according to Ministry guidance. If a company does not follow the guidelines, it can be penalized under the law. Figure 4.6 shows the decision tree for the practical test used to monitor the allergy-labeling system. Local governments and health centers monitor labeling according to this decision tree. Incorrect labeling of specific allergenic ingredients on the processed food products has occurred. Such errors should be corrected using Ministry guidance.

G. Development of detection methods for subspecific allergenic ingredients

1. Soybean

In 1999, the Joint FAO/WHO Codex Alimentarius Commission agreed to recommend the labeling of eight food ingredients, including soybean, which are known to be allergens (FAO, 1995; Hefle *et al.*, 1996). As soybean is one of the "big eight" ingredients believed to be responsible for 90% of all food allergies (Zarkadas *et al.*, 1999), it is recommended that labeling is an important issue, more so because of the almost unlimited use of soybean and the increasing number of patients who are allergic soybeans (Bock and Atkins, 1990; Foucard and Malmheden Yman, 1999; Sampson, 2001; Sicherer *et al.*, 2000).

We therefore developed an ELISA for the detection of soybean protein in processed foods using polyclonal antibodies raised against p34 as a soybean marker protein and using a specific extraction buffer (Morishita *et al.*, 2008). The p34 protein, originally characterized as an oil

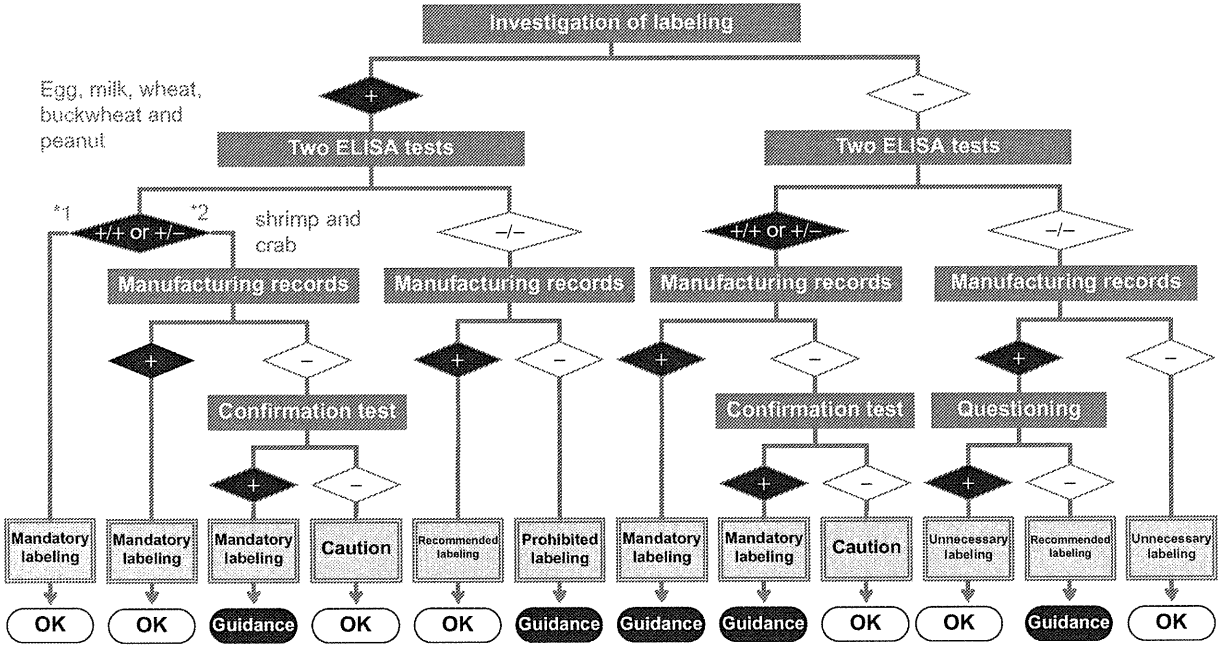

FIGURE 4.6 Decision tree for the practical test used to monitor the allergy-labeling system.

body-associated protein in soybean, has been identified as one of the major allergenic proteins and named Gly m Bd 30K. Our ELISA method is highly specific for this soybean protein, with the LOD of 0.47 ng/mL (equivalent to 0.19 μg/g food) and limit of quantification (LOQ) of 0.94 ng/mL (equivalent to 0.38 μg/g food). Recovery ranged from 87.7% to 98.7%, while the intra- and interassay coefficients of variation were less than 4.2% and 7.5%, respectively. These results show that this ELISA method is specific, precise, and reliable for quantitative analysis of the soybean protein in processed foods. Five types of incurred samples (model processed foods: rice gruel, sausage, sweet adzuki-bean soup, sweet potato cake, and tomato sauce) containing 10 μg soybean soluble protein/g food were prepared for use in interlaboratory evaluations of the soybean ELISA kit (Sakai *et al.*, 2009). The kit displayed a sufficient RSD_r value (interlaboratory precision: 9.3–13.4% RSD_r) and a high recovery (97–114%) for all incurred samples. The RSD_r value for the incurred samples was mostly $<4.8\%$. The results of this interlaboratory evaluation suggest that the soybean kit can be used as a precise and reliable tool for determination of soybean proteins in processed foods.

A sensitive qualitative detection method for soybeans in foods using PCR was also developed (Yamakawa *et al.*, 2007b). For specific detection of soybeans with high specificity, the primer pair was designed using the gene encoding the *Glycine max* repetitive sequence. Trace amounts of soybeans in commercial food products could be qualitatively detected by this method.

2. Walnut

Tree nuts are regarded as one of the most potent of all known food allergens and are often attributed as the cause of severe food anaphylaxis and death. Walnut (*Juglans regia*) is the most common allergenic tree nut and this allergy can be observed in all age groups (Bock *et al.*, 2001). In addition, the walnut allergy is extremely potent, inducing life threatening allergic reactions similar to peanut allergy (Clark and Ewan, 2003; Pumphrey, 2000; Pumphrey and Roberts, 2000). According to Japanese regulations, the labeling of food products containing walnut is recommended. To ensure proper labeling, a novel sandwich ELISA kit for the determination of walnut protein in processed foods has been developed (Doi *et al.*, 2008). The sandwich ELISA method is highly specific for walnut soluble proteins. The recovery ranged from 83.4% to 123%, while the intra- and interassay coefficients of variation were less than 8.8% and 7.2%, respectively. We prepared seven types of incurred samples (model processed foods: biscuit, bread, sponge cake, orange juice, jelly, chicken meatball, and rice gruel) containing 10 μg walnut soluble protein/g food for use in interlaboratory evaluations of the walnut ELISA method (Sakai *et al.*, 2010a). The walnut kit displayed a sufficient RSD_r

(interlaboratory precision: 5.8–9.9% RSD_r) and a high level of recovery (81–119%) for all the incurred samples. All RSD_r values for the incurred samples examined were less than 6.0%. The results of this interlaboratory evaluation suggest that the walnut ELISA method can be used as a precise and reliable tool for determination of walnut proteins in processed foods.

A sensitive qualitative detection method for walnut using PCR was also developed (Yano *et al.*, 2007). For detection of walnuts with high specificity, the primer pair was designed based on walnut *matK* genes. Trace amounts of walnuts in commercial food products can be qualitatively detected using this method.

3. Kiwifruit

Kiwifruit (*Actinidia deliciosa* and *A. chinensis*) is a major fruit allergen that produces severe symptoms and is responsible for a large number of clinical cases worldwide (Lucas *et al.*, 2003; Lucas *et al.*, 2004; Möller *et al.*, 1998a). Under Japanese regulations, it is recommended for labeling as much as possible. To develop PCR-based methods for detection of trace amounts of kiwifruit in foods, we designed two primer pairs targeting the ITS-1 region of the *Actinidia* spp. using PCR simulation software (Taguchi *et al.*, 2007). On the basis of the known distribution of a major kiwifruit allergen (actinidin) within the *Actinidia* spp., in addition to reports on clinical and immunological cross-reactivities, one of the primer pairs was designed to detect all *Actinidia* spp. and the other to detect commercially grown *Actinidia* spp. (i.e., *A. arguta* and is interspecific hybrids) except for *A. polygama*. The specificity of these methods using designed primer pairs was verified by PCR on eight *Actinidia* spp. and 26 other plants, including fruits. The methods were considered to be specific enough to yield products of the target-size only from *Actinidia* spp. and sensitive enough to detect 5–50 fg of *Actinidia* spp. DNA spiked in 50 ng salmon testis DNA used as a carrier (1–10 ppm of kiwifruit DNA) and 1700 ppm (wt/wt) of fresh kiwifruit puree spiked in a commercial plain yogurt (corresponded to ca. 10 ppm of kiwifruit protein). These methods are expected to be useful in the detection of unidentified kiwifruit and its related species in processed foods.

4. Banana

Banana contains food allergens that are common to those in latex or pollens (Ito *et al.*, 2006; Sanchez-Monge *et al.*, 1999). Many clinical studies have reported cross-reactivity of banana and latex, referred to as the latex-fruit syndrome (Blanco *et al.*, 1999; Ikezawa and Osuna, 2002; Möller *et al.*, 1998b). These studies monitored the number of patients with food allergy in Japan and found that patients with banana allergy comprised the second largest population (below only kiwifruit allergy) among those with fruit allergies. We developed specific PCR methods for detection of

banana residue in processed foods. For high banana specificity, the primer pair was designed based on the large subunit of ribulose-1, 5-bisphosphate carboxylase (*rbcL*) genes of chloroplasts and used to obtain amplified products specific to banana by both conventional and real-time PCR (Sakai *et al.*, 2010b). To confirm the specificity of these methods, genomic DNA samples from 31 other species were examined; no amplification products were detected. Subsequently, eight kinds of processed foods containing banana were investigated using these methods to confirm the presence of banana DNA. Conventional PCR had a detection limit of 1 ppm (wt/wt) banana DNA spiked in 50 ng of salmon testis DNA, while SYBR Green I real-time semi-quantitative PCR had a detection limit as low as 10 ppm banana DNA. Thus, both methods show high sensitivity and may be applicable as specific tools for the detection of trace amounts of banana in commercial food products.

5. Pork, beef, chicken, mutton, and horseflesh

As the modern diet often comprises processed foods, especially minced meats, manufacturers are obligated to properly label raw materials. Hence, a rapid method of detecting meat ingredients in processed foods is needed to verify proper labeling. A rapid real-time quantitative PCR method to detect trace amounts of pork, beef, chicken, mutton, and horse meat in foods was developed (Tanabe *et al.*, 2007). The primers and TaqMan minor groove binder (MGB) probes were designed using the gene encoding cytochrome *b* for specific detection of each species. The LOQ of this method was 100 fg/μL of each mitochondrial DNA in 10 ng/μL of wheat mitochondrial DNA matrix. The calculated R^2 values of the standard curves for the five species ranged between 0.994 and 0.999. This method is particularly useful in the detection of unidentified minced meat in processed foods for verification of food labeling.

IV. PATIENT EVALUATION OF ALLERGY FOOD LABELING

To clarify the usefulness and reliability of the food-labeling system, food allergy patients (or their parents) at Sagamihara National Hospital were asked to evaluate it by questionnaire. We received responses from 169 patients. As shown in Table 4.20, patients' profiles were an average age of 49.3 ± 35.6 months, age of the first onset of symptoms of 10.1 ± 14.1 months, and average of 2.9 ± 2.5 eliminated foods. Eliminated foods included hen's eggs (135), cow's milk (79), and wheat (47), as well as peanuts and fish eggs. Of these patients, 44.2% had a past history of anaphylaxis, and 80.2% had experienced symptoms following exposure to even extremely small amounts of the causative foods.

TABLE 4.20 Characteristics of surveyed subjects

169 parents of food allergy patients at Sagamihara National Hospital	
Age of patients	49.3 ± 35.6 months M/F = 1.9
Age of first onset of symptom	10.1 ± 14.1 months
Number of eliminated foods	2.9 ± 2.5
Eliminated foods	
Hen's eggs	135
Cow's milk	79
Wheat	47
Peanuts	51
Fish eggs	28
Past history of anaphylaxis	44.2%
Incidence of symptom by extremely small amount	80.2%

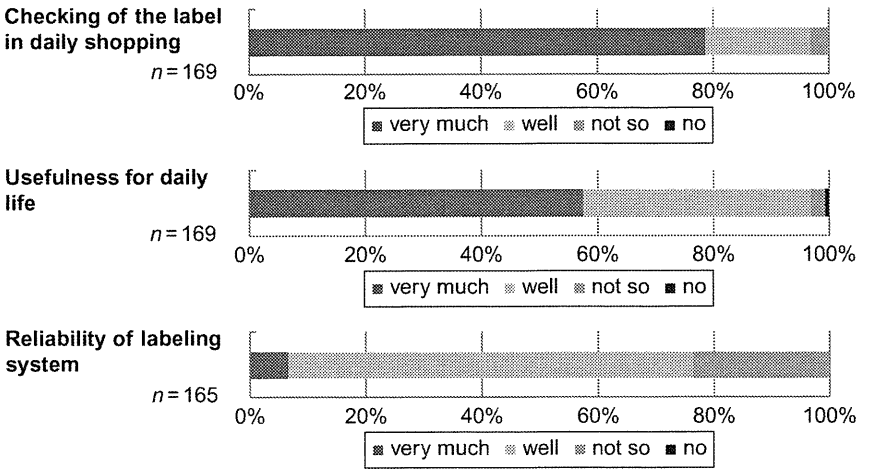

FIGURE 4.7 Evaluation of allergy food labeling.

As shown in Fig. 4.7, 97% of patients routinely checked the allergy food label during daily shopping, and 97% evaluated the allergy food labeling as "very useful" or "useful." In addition, 76.4% of the respondents relied on the allergy food-labeling system, and 79.3% had a correct understanding of the food-labeling system based on self-evaluation. On the other hand, 48.8% of respondents answered that the labeling system was "very easy" or "easy" to understand (Fig. 4.8). Patients who had experienced accidental intake by misreading a label or by mislabeling comprised 30.9% and 13.9%, respectively (Fig. 4.9).

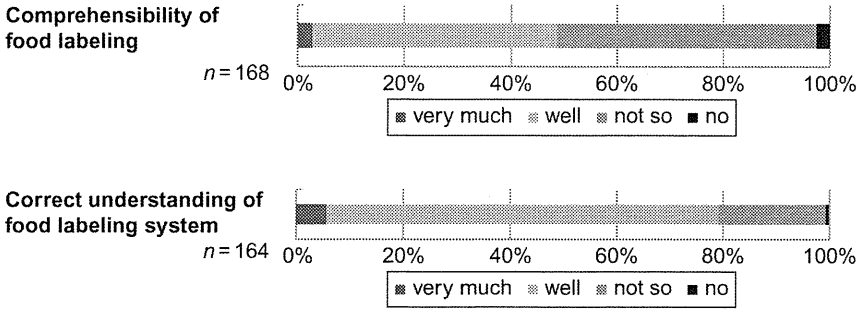

FIGURE 4.8 Comprehension and understanding of allergy food labeling.

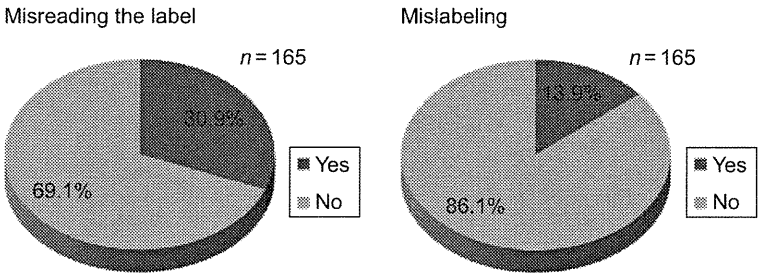

FIGURE 4.9 Incidences of accidental intake by misreading and mislabeling of food labels.

Overall, the Japanese food allergy-labeling system was highly evaluated by food allergy patients and parents. Almost all patients felt that the food-labeling system was very useful, although there were cases of accidental intake either by misreading the label or by mislabeling by food companies.

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REFERENCES

Blanco, C., Diaz-Perales, A., Collada, C., Sánchez-Monge, R., Aragoncillo, C., Castillo, R., Ortega, N., Alvarez, M., Carrillo, T., and Salcedo, G. (1999). Class I chitinases as potential panallergens involved in the latex-fruit syndrome. *J. Allergy Clin. Immunol.* **103**, 507–513.

Bock, S. A. and Atkins, F. M. (1990). Patterns of food hypersensitivity during sixteen years of double-blind, placebo-controlled food challenges. *J. Pediatr.* **117**, 561–567.

Bock, S. A., Munoz-Furlong, A., and Sampson, H. A. (2001). Fatalities due to anaphylaxis reaction to foods. *J. Allergy Clin. Immunol.* **107**, 191–193.

Clark, A. T. and Ewan, P. W. (2003). Interpretation of tests for nut allergy in one thousand patients, in relation to allergy or tolerance. *Clin. Exp. Allergy* **33**, 1041–1045.

Doi, H., Shibata, H., Shoji, M., Sakai, S., and Akiyama, H. (2008). A reliable enzyme linked immuno-sorbent assay for the determination of walnut proteins in processed foods. *J. Agric. Food Chem.* **56**, 7625–7630.

Ebisawa, M., Ikematsu, K., Imai, T., and Tachimoto, H. (2003). *J. World Allergy Organiz.* **15**, 214–217.

Food and Agriculture Organization of the United Nations, World Health Organization (FAO (1995). Report of FAO technical consultation on food allergens. Rome 1995, November, 13–14.

Foucard, T. and Malmheden Yman, I. (1999). A study on severe food reactions in Sweden—Is soy protein an underestimated cause of food anaphylaxis? *Allergy* **54**, 261–265.

Hefle, S. L., Nordlee, J. A., and Taylor, S. L. (1996). Allergenic foods. *Crit. Rev. Food Sci. Nutr. Allergy Clin. Immunol.* **36**(Suppl.), 69–89.

Ikezawa, Z. and Osuna, H. (2002). Latex-fruits syndrome. *Arerugi* **51**, 591–604 (in Japanese).

Ito, A., Ito, K., Morishita, M., and Sakamoto, T. (2006). A banana-allergic infant with IgE reactivity to avocado, but not to latex. *Pediatr. Int.* **48**, 321–323.

Lucas, J. S. A., Lewis, S. A., and Hourihane, J. O. (2003). Kiwi fruit allergy: A review. *Pediatr. Allergy Immunol.* **14**, 420–428.

Lucas, J. S. A., Grimshaw, K. E. C., Collins, K., Warner, J. O., and Hourihane, J. O. (2004). Kiwi fruit is a significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin. Exp. Allergy* **34**, 1115–1121.

Matsuda, R., Yoshioka, Y., Akiyama, H., Aburatani, K., Watanabe, Y., Matsumoto, T., Morishita, N., Sato, H., Mishima, T., Gamo, R., Kihira, Y., and Maitani, T. (2006). Inter-laboratory evaluation of two kinds of ELISA kits for the detection of egg, milk, wheat, buckwheat, and peanut in foods. *J. AOAC Int.* **89**, 1600–1608.

Möller, M., Kayma, M., Vieluf, D., Paschke, A., and Steinhart, H. (1998). Determination and characterization of cross-reacting allergens in latex, avocado, banana, and kiwi fruit. *Allergy* **53**, 289–296.

Morishita, N., Kamiya, K., Matsumoto, T., Sakai, S., Teshima, R., Urisu, A., Moriyama, T., Ogawa, T., Akiyama, H., and Morimatsu, F. (2008). A reliable enzyme-linked immuno-sorbent assay for determination of soybean proteins in processed foods. *J. Agric. Food Chem.* **56**, 6818–6824.

Notification No. 1106001 of November 6, 2002, and the revised Notification No. 0622003 of June 22, 2006, Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan.

Pumphrey, R. S. H. (2000). Lessons for management of anaphylaxis from a study of fatal reaction. *Clin. Exp. Allergy* **30**, 1144–1150.

Pumphrey, R. S. H. and Roberts, I. S. D. (2000). Postmortem findings after fatal anaphylactic reactions. *J. Allergy Clin. Pathol.* **53**, 273–276.

Sakai, S., Matsuda, R., Adachi, R., Akiyama, H., Maitani, T., Ohno, Y., Oka, M., Abe, A., Seiki, K., Oda, H., Shiomi, K., and Urisu, A. (2008). Interlaboratory evaluation of two enzyme-linked immunosorbent assay kits for the determination of crustacean protein in processed foods. *J. AOAC Int.* **91**, 123–129.

Sakai, S., Adachi, R., Akiyama, H., Teshima, R., Morishita, N., Matsumoto, T., and Urisu, A. (2009). Interlaboratory evaluation of an enzyme-linked immunosorbent assay kit for the determination of soybean protein in processed foods. *J. AOAC Int.* **93**, 243–248.

Sakai, S., Adachi, R., Akiyama, H., Teshima, R., Doi, H., and Shibata, H. (2010a). Determination of walnut protein in processed foods by enzyme-linked immunosorbent assay interlaboratory study. *J. AOAC Int.* **93**, 1255–1261.

Sakai, Y., Ishihata, K., Nakano, S., Yamada, T., Yano, T., Uchida, K., Nakao, Y., Urisu, A., Adachi, R., Teshima, R., Akiyama, H., Sakai, Y., *et al.* (2010b). Specific detection of banana residue in processed foods using polymerase chain reaction. *J. Agric. Food Chem.* **58**, 8145–8151.

Sampson, H. A. (2001). Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J. Allergy Clin. Immunol.* **107**, 891–896.

Sanchez-Monge, R., Blanco, C., Diaz-Perales, A., Collada, C., Carrillo, T., Aragoncillo, C., and Salcedo, G. (1999). Isolation and characterization of major banana allergens: Identification as fruit class I chitinases. *Clin. Exp. Allergy* **29**, 673–680.

Seiki, K., Oda, H., Yoshioka, H., Sakai, S., Urisu, A., Akiyama, H., and Ohno, Y. (2007). A reliable and sensitive immunoassay for the determination of crustacean protein in processed foods. *J. Agric. Food Chem.* **55**, 9345–9350.

Shibahara, Y., Oka, M., Tominaga, K., Ii, T., Umeda, M., Uneo, N., Abe, A., Ohashi, E., Ushio, H., and Shiomi, K. (2007). Determination of crustacean allergen in food products by sandwich ELISA. *Nippon Shokuhin Kagaku Kogaku Kaishi* **54**, 280–286.

Sicherer, S. H., Sampson, H. A., and Burks, A. W. (2000). Peanut and soy allergy: A clinical and therapeutic dilemma. *Allergy* **55**, 515–521.

Taguchi, H., Watanabe, S., Hirao, T., Akiyama, H., Sakai, S., Watanabe, T., Matsuda, R., Urisu, A., and Maitani, T. (2007). Specific detection of potentially allergenic kiwifruit in foods using polymerase chain reaction. *J. Agric. Food Chem.* **55**, 1649–1655.

Tanabe, S., Hase, M., Yano, T., Sato, M., Fujimura, T., and Akiyama, H. (2007). Real-time quantitative PCR detection method for pork, chicken, beef, mutton, and horseflesh in foods. *Biosci. Biotechnol. Biochem.* **71**, 3131–3135.

Watanabe, K., Aburatani, T., Mizumura, M., Sakai, H., Muraoka, S., Mamegoshi, S., and Honjoh, T. (2005). Novel ELISA for the detection of raw and processed egg using extraction buffer containing a surfactant and a reducing agent. *J. Immunol. Methods* **300**, 115–123.

Watanabe, T., Akiyama, H., Yamakawa, H., Iijima, K., Yamazaki, F., Matsumoto, T., Futo, S., Arakawa, F., Watai, M., and Maitani, T. (2006). A specific qualitative detection method for peanut (*Arachis hypogaea*) in foods using polymerase chain reaction. *J. Food Biochem.* **30**, 215–233.

Yamakawa, H., Akiyama, H., Endo, Y., Miyatake, K., Sakata, K., Sakai, S., Toyoda, M., and Urisu, A. (2007a). Specific detection of wheat residues in processed foods by polymerase chain reaction. *Biosci. Biotechnol. Biochem.* **71**, 2561–2564.

Yamakawa, H., Akiyama, H., Endo, Y., Miyatake, K., Sakata, K., Sakai, S., Moriyama, T., Urisu, A., and Maitani, T. (2007b). A specific detection of soybean residues in processed foods using polymerase chain reaction. *Biosci. Biotechnol. Biochem.* **71**, 269–272.

Yamakawa, H., Akiyama, H., Endo, Y., Miyatake, K., Sakata, K., Sakai, S., Toyoda, M., and Urisu, A. (2008). Specific detection of buckwheat residues in Processed foods by polymerase chain reaction. *Biosci. Biotechnol. Biochem.* **72**, 2228–2231.

Yano, T., Sakai, Y., Uchida, K., Nakao, Y., Ishihata, K., Nakano, S., Yamada, T., Sakai, S., Urisu, A., Akiyama, H., and Maitani, T. (2007). Detection of walnut residues in processed foods by polymerase chain reaction. *Biosci. Biotechnol. Biochem.* **71**, 1793–1796.

Zarkadas, M., Scott, W. F., Salminen, J., and Pong, A. H. (1999). Common allergenic foods and their labelling in Canada. *Can. J. Allergy Clin. Immunol.* **4**, 118–141.

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Clinical Utility of IgE Antibodies to ω-5 Gliadin in the Diagnosis of Wheat Allergy: A Pediatric Multicenter Challenge Study

Motohiro Ebisawa[a] Rumiko Shibata[b] Sakura Sato[a] Magnus P. Borres[d, e] Komei Ito[c]

[a]Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Sagamihara, [b]Department of Pediatrics, Fukuoka National Hospital, Fukuoka, and [c]Department of Allergy, Aichi Children's Health and Medical Center, Obu, Japan; [d]Medical Department, Phadia AB, Uppsala, and [e]Institute for Clinical Sciences, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Key Words
Allergy tests · Food challenge · IgE · ω-5 gliadin · Pediatric allergy · Wheat

Abstract
Background: There are contradictory results regarding the clinical usefulness of the determination of IgE antibodies to ω-5 gliadin in children with a suspicion of wheat allergy (WA). ***Methods:*** The study comprised 311 children and young adults with suspected wheat intolerance treated at three separate pediatric clinics and, with the exception of 25, were found to be positive in specific IgE antibody determinations to wheat. Their ages ranged from 6 months to 20.4 years (median age, 2.3 years). Possible relationships between IgE antibodies to ω-5 gliadin and a physician's diagnosis of WA and challenge symptoms were studied. ***Results:*** The mean concentration of IgE antibodies to ω-5 gliadin was 1.2 kU_A/l in WA patients and <0.35 kU_A/l in patients without WA ($p < 0.0001$). Seventy-two percent of the WA patients had positive ω-5 gliadin levels and 75% of the patients without WA had negative levels. Logistic regression showed a significant relationship between the probability of WA and the concentration of IgE antibodies to ω-5-gliadin with a 2.6-fold (95% CI: 2.0–3.3) increased risk. Age was an important factor to consider as the risk of WA increased 5.4-fold (95% CI: 1.4–21) for children ≤1 year of age and 2.5-fold (95% CI: 2.0–3.2) for children >1 year of age with increasing levels of IgE. ***Conclusion:*** Detection of IgE to ω-5 gliadin seems to be associated with ~~lack of~~ responsiveness to the challenge test and is particularly useful in infants with a suspicion of WA.

Introduction

Wheat is one of the six most common foods causing allergy in children and the third most common food allergen in Japanese children [1]. IgE antibodies to egg, cow's milk and peanut have been useful in predicting clinical reactivity [2–4]. Furthermore, the correlation between the outcome of oral food challenges and levels of IgE antibodies to wheat was established in Japanese children with suspected wheat allergy (WA) [5]. ω-5 gliadin has been identified as the major antigen in children with wheat-dependent, exercised-induced anaphylaxis [6]. Furthermore, IgE antibodies to ω-5 gliadin have been found in children with immediate reactions to ingested wheat [7], and recently published results show that increased levels of IgE antibodies to ω-5 gliadin correlate with the outcome of oral

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Correspondence to: Dr. Motohiro Ebisawa
Clinical Research Center for Allergy and Rheumatology
Sagamihara National Hospital, 18-1, Sakuradai
Sagamihara, Kanagawa 228-8522 (Japan)
Tel. +81 42 742 8311, E-Mail m-ebisawa@sagamihara-hosp.gr.jp

wheat challenge [8]. The level of antibodies to ω-5 gliadin has thus been suggested to serve as a marker for clinical reactivity and an aid for the decision for or against wheat challenge. Contradictory results have been reported from two populations of German and American children where no correlation between ω-5 gliadin antibody levels and the outcome of oral food challenge could be demonstrated [9].

The objective of this survey was to evaluate the possible clinical usefulness of IgE antibody concentrations to ω-5 gliadin in relation to WA in a large cohort of food-challenged children.

Patients and Methods

This retrospective study was performed in 311 children and young adults with suspected wheat intolerance treated at three different clinics in Japan. The clinics were the Fukuoka National Hospital (n = 88, site 1), Fukuoka, the Aichi Children's Hospital (n = 114, site 2), and the Ohbu and Sagamihara National Hospital (n = 109, site 3), Sagamihara. The three participating departments were representative for pediatric allergology in Japan. Inclusion criteria were suspicion of WA based either on clinical history and/or serology. All individuals, except for 25, had specific IgE antibody to wheat. The children had been referred from primary care physicians or enrolled at the outpatient clinic due to immediate hypersensitivity reactions following wheat ingestion. Clinical history comprised skin, gastrointestinal tract and/or respiratory tract symptoms following wheat ingestion. The age of the patients ranged from 6 months to 20.4 years (median age, 2.3 years), and 215 of the individuals were males. Informed consent was given by the child or child's parents prior to enrolment.

Clinically, each patient was subjected to a detailed medical examination and medical history data were collected. In order to confirm or exclude WA, the diagnosis was based on either oral food challenges or case history or, in most cases, a combination of both. Blood was sampled for baseline determination of IgE antibodies to wheat and ω-5 gliadin. Serum samples were analyzed for IgE antibodies to wheat and ω-5 gliadin using the ImmunoCAP® System FEIA (Phadia AB, Uppsala, Sweden). The detection limit of the assays was 0.35 kU_A/l.

All wheat-food challenges were open challenges performed in a hospital setting and supervised by physicians in accordance with the guidelines for the diagnosis and management of pediatric food allergy in Japan [10, 11]. In children with a strong convincing history for high-risk responses, including severe symptoms on challenge, the challenge procedure was not carried out (n = 36). When a child had no objective WA symptoms and/or was eating wheat (n = 60), a challenge was not carried out and the child was classified as no WA (NoWA). Based on case history, physical examination and, in most cases, challenge outcome, each child was classified as having an immediate hypersensitivity reaction to ingested wheat or NoWA.

Statistical Methods

The Mann-Whitney non-parametric test was used to test differences between the groups. A p value of 0.05 was regarded as significant. Performance characteristics, i.e. sensitivity and specificity, were calculated for various cutoff values, including the optimal cutoff values proposed by the receiver-operating characteristic (ROC) plots. For quantitative evaluation, a logistic regression model was formulated as the probability of receiving a positive clinical diagnosis as a function of the logarithm of the specific IgE concentration:

$$\text{logit}(Pr[Y = 1/\ln \text{IgE (kU/l)}]) = \alpha + \beta \ln \text{IgE (kU/l)}$$

This quantitative model describes the relationship between sensitization and clinical diagnosis of WA. Statistical analysis was carried out using SAS System V9.1.

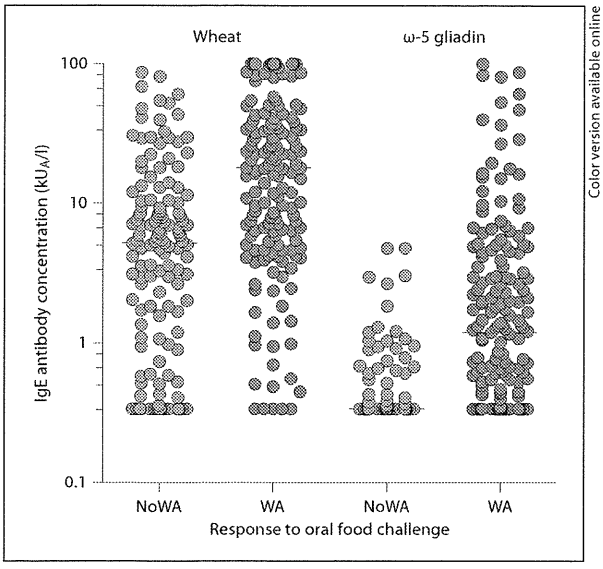

Fig. 1. Allergen-specific IgE titers for wheat and ω-5 gliadin for WA and NoWA patients.

Results

The diagnosis of WA was confirmed in 173 children by symptoms following oral wheat challenge or a convincing case history of anaphylaxis in relation to wheat intake. Five of these children had specific IgE to wheat <0.35 kU_A/l. The remaining 138 children were classified as NoWA. The demographic characteristics and outcome of oral wheat challenges are presented in table 1 for both WA and NoWA patients. The median concentrations of IgE antibodies to wheat and ω-5 gliadin in children with confirmed WA were 18.1 (range <0.35 to >100) and 1.2 (range <0.35–100) kU_A/l, respectively. The corresponding values

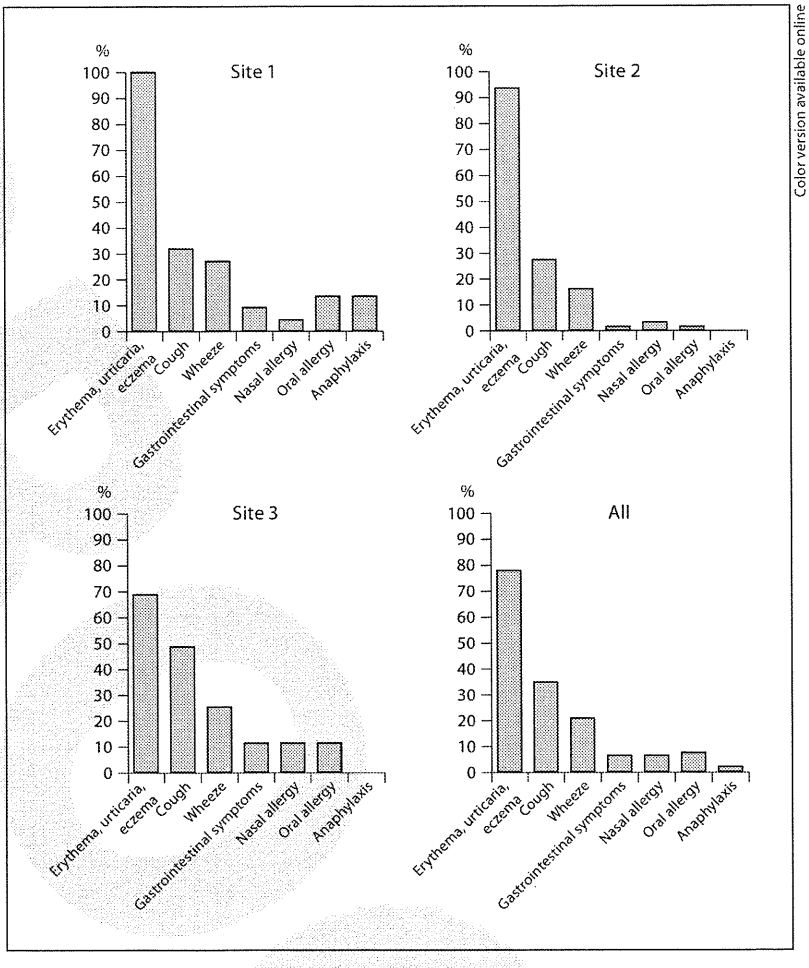

Fig. 2. Symptom frequencies in WA patients at food challenge. Numbers of patients are given in table 1.

for the children classified as NoWA were 5.2 (range <0.35–86.8) and <0.35 (range <0.35–4.8) kU_A/l, respectively (fig. 1). This difference in allergen-specific IgE concentrations between the two groups was significant ($p < 0.0001$). Seventy-two percent (125 of 173) of the WA patients had positive ω-5 gliadin levels and 75% (104 of 138) of the NoWA patients had negative ω-5 gliadin levels. In total, 137 of the 173 WA patients were challenged, and symptom responses following challenge are presented in figure 2 for each of the clinics separately and for all three combined. Skin reactions (erythema, urticaria and eczema) were predominating, amounting to >75%. As shown for site 1, >10% of the patients experienced an anaphylactic reaction during the challenge. In the remaining WA patients that were not challenged but had a convincing history (n = 36), skin reactions (94%), cough (17%), wheezing (14%) and anaphylaxis (5%) were the most frequent symptoms.

ROC analyses were performed to evaluate the diagnostic ability of the in vitro tests. The area under the curve (AUC) for ω-5 gliadin was 78.5% (fig. 3). At an estimated cutoff of 0.41 kU_A/l, values of 72% (sensitivity), 79% (specificity), 81% (PPV), 69% (NPV), 3.4 (positive likelihood ratio) and 0.5 (negative likelihood ratio) were obtained for the assay. The corresponding figures for wheat were: AUC 73.0%, 61% (sensitivity), 74% (specificity), 75% (PPV), 60% (NPV), 2.4 (positive likelihood ratio) and 0.5 (negative likelihood ratio) at a cutoff of 10.1 kU_A/l. The concentration difference between the two pediatric groups was further investigated using a logistic regression model. A significant relationship between the probability of WA and

Table 1. Demographic and background characteristics of the patients (numbers, medians and ranges)

		Wheat allergy			No wheat allergy		
		sex	age, years	challenge/ convincing history	sex	age, years	challenge/ convincing history
Site 1	Male (n = 79)	31	2.4 (1.0–8.7)		48	3.9 (1.0–20.4)	
	Female (n = 35)	14	3.5 (1.0–7.1)	22/23	21	4.5 (1.4–15.4)	
	Total (n = 114)						24/45
Site 2	Male (n = 62)	48	1.6 (0.6–7.1)		14	1.9 (0.8–7.6)	
	Female (n = 26)	19	2.1 (0.6–8.8)		7	3.0 (0.9–6.9)	
	Total (n = 88)			63/4			21/0
Site 3	Male (n = 71)	38	2.8 (1.1–12.0)		33	1.8 (0.6–14.8)	
	Female (n = 38)	23	2.3 (0.8–8.6)		15	2.1 (0.5–8.2)	
	Total (n = 109)			52/9			33/15
All sites	Male (n = 212)	117	2.0 (0.6–12.0)		95	2.8 (0.6–20.4)	
	Female (n = 99)	56	2.2 (0.6–8.8)		43	3.3 (0.5–15.4)	
	Total (n = 311)			137/36			78/60

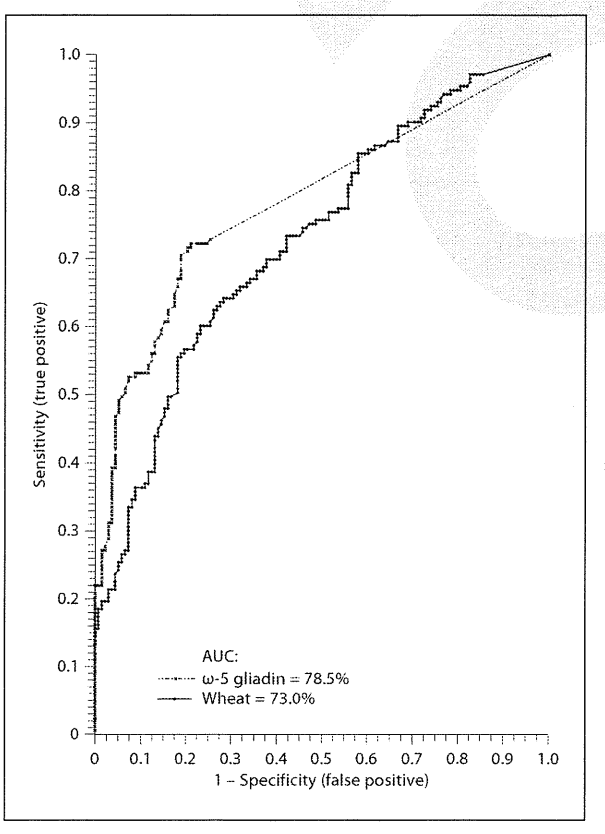

Fig. 3. ROC curve based on all patients.

the concentration of IgE antibodies to ω-5 gliadin was thus found. The risk increased 2.6-fold (95% CI: 2.0–3.3) with increasing levels of IgE (fig. 4a). Furthermore, when grouping the children into different age groups, there was a significant difference between ≤1 and >1 year of age in the probability of WA for the concentration of IgE antibodies to ω-5 gliadin. The risk of WA increased for children ≤1 year of age 5.4-fold (95% CI: 1.4–21) and 2.5-fold (95% CI: 2.0–3.2) for children >1 year of age with increasing levels of IgE, as illustrated in figure 4a. The relationship between the concentration of IgE antibodies to wheat and ω-5 gliadin was also investigated in a logistic regression model. A significant association to WA was found for ω-5 gliadin with a 2.1-fold increased risk (95% CI: 1.9–3.6). Figure 4b shows the probability of WA at nine different values of ω-5 gliadin increased with increasing antibody concentrations of specific IgE to wheat.

Discussion

Our results confirmed a difference in the IgE levels of ω-5 gliadin between WA and NoWA children in this multicenter challenge study. Further, we demonstrate that IgE antibodies to ω-5 gliadin are particularly useful in predicting food challenge outcome in children <1 year of age.

Our findings based on 311 Japanese children are not in agreement with the recent findings of Beyer et al. [9], who could not find a correlation between ω-5 gliadin lev-