

Egg and Milk Proteins as Hidden Allergens in Food: 5-Year (2010 to 2014) Results of Food Allergen Monitoring in Piedmont, Italy

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ABSTRACT

Cow's milk and egg allergies are two of the most common food allergies. Manufacturers of food products containing milk or eggs or their derivatives as an ingredient are required by European Union regulations to list their presence on the ingredient label. Under European Union legislation, member states are mandated to carry out food safety monitoring programs to verify compliance with food labeling requirements. Through the Regional Integrated Plan for Food Safety, the Piedmont (Italy) regional authority carries out an annual program to determine the presence of undeclared allergens in foods. In the 5-year period from 2010 to 2014, a total of 1,566 food samples were analyzed for the presence of hidden egg and milk proteins. The average positive percentage was 2.8% (3.6% egg and 2% milk proteins). Comparison between the allergen concentration and the published eliciting dose (ED) for egg proteins (0.03 mg) and for total milk proteins (0.1 mg) indicated a high risk of allergen exposure for sensitized consumers. The calculated exposure was up to 135× (for milk) the ED01 reported in the literature. Food manufacturers will need to improve their allergen control programs to reduce allergen exposure and risk.

Key words: Consumer exposure; Egg; Food allergens; Milk; Official control; Threshold

The prevalence of food allergies is increasing, particularly among younger children (13). Food allergies affect between 2 and 4% of the population in developed countries (16), with a prevalence of up to 8% in children (13). Cow's milk is one of the most common food allergens worldwide, affecting approximately 3% of infants and young children (8, 12). The prevalence of egg allergy is estimated to be 2% in children and 0.1% in adults (3). Exposure to milk and egg protein is increased owing to the presence of their derivatives (e.g., milk or egg powder, caseins, or lysozyme) in scores of food products. Because allergy to foodstuffs can cause severe and sometimes life-threatening reactions, food allergen labeling is critical for ensuring that consumers make safe choices in the food they purchase.

To protect allergic consumers, European legislation requires the provision of allergen information on labels so that food-allergic consumers can avoid foods that trigger their allergic reactions. Under European Regulation No 1169/2011 (5), the presence of allergens in a food product must be declared in the ingredients list on food labels. At the time of this writing, Annex II of EU Regulation 1169/2011 identifies 14 declarable allergens: cereals containing gluten, crustaceans, mollusks, eggs, fish, peanuts, tree nuts, soybeans, milk, celery, mustard, sesame, lupin, and sulfur

dioxide at levels above 10 mg/kg, or 10 mg/liter, expressed as SO₂.

Under European Union legislation, member states are mandated to carry out food safety monitoring programs to verify compliance with food labeling requirements. Unlike the legal status of microbiological criteria for food safety, European legislation does not specify a method official laboratories should use for determining compliance, nor does it indicate which international standard organization protocols are to be applied in official food allergen investigations. As a result of this regulatory vacuum, many different analytical approaches are employed to investigate the presence of food allergens (10). Enzyme-linked immunosorbent assay (ELISA)-based methods, because they allow for ease of processing at low cost of laboratory equipment and materials, are one of the most widely techniques (6, 11). However, the use of high temperature or high pressure industrial practices or both can cause the partial (or complete) denaturation of proteins in foods, considerably reducing the sensitivity of sample testing (2).

Alternative approaches to evaluate the safety of a certain food in relationship to the presence of allergens rely on PCR-based methods that use DNA as a marker of allergenic proteins. The drawback to these methods is that many substances commonly present in foods can cause inhibition (7) or cross-reactivity of the food matrix when animal origin allergens (milk or eggs) are tested in food

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TABLE 1. Number of samples planned, collected, and delivered to the laboratory for egg protein and beta-lactoglobulin detection during the 5-year period 2010 to 2014^a

	PRISA samples (planned)	PRISA samples (collected)	Foodborne illness samples	Consumers complaint samples	Total no. of samples	No. (%) of positive samples
Egg proteins						
2010	100	207 (+107%)	0	42	249	7 (2.8)
2011	100	173 (+73%)	4	6	183	8 (4.4)
2012	70	133 (+90%)	1	21	155	8 (5.2)
2013	40	65 (+63%)	0	19	84	3 (3.6)
2014	40	76 (+90%)	1	27	104	2 (1.9)
Total egg proteins	350	654	6	115	775	28 (3.6)
Beta-lactoglobulin						
2010	100	214 (+114%)	0	35	249	1 (0.4)
2011	100	140 (+40%)	1	14	155	7 (4.5)
2012	50	162 (+224%)	1	21	184	8 (4.3)
2013	60	85 (+42%)	0	18	103	0
2014	60	73 (+22 %)	1	26	100	0
Total beta-lactoglobulin	370	674	3	114	791	16 (2.0)
Total	720	1,328	9	229	1,566	44 (2.8%)

^a PRISA, Regional Integrated Plan for Food Safety (Piano Integrato per la Sicurezza Alimentare).

derived from the same animal (red meat or chicken meat preparations, respectively).

Through the Regional Integrated Plan for Food Safety (Piano Integrato per la Sicurezza Alimentare [PRISA]), the Piedmont (Italy) regional competent authority carries out annual sampling to verify that food labels comply with national and European regulatory requirements. The sampling program for monitoring undeclared allergens in foods has been in place since 2007 (4). Samples are delivered to the Food Safety Control Department of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy. Since October 2013, they are delivered to the Regional Center for Food Intolerance and Allergies. In addition to samples from routine monitoring, the laboratory also receives and analyzes food samples in response to reports of customer complaints and suspected foodborne outbreaks, including illnesses caused by allergens. This study reports the 5-year results of the sampling program in which several food matrices were investigated for the presence of hidden milk (beta-lactoglobulin) and egg (total egg proteins) allergens.

MATERIALS AND METHODS

Sample collection. Samples were randomly collected from retail food stores and supermarkets throughout northwest Italy from January 2010 through December 2014. Both animal origin foods and vegetable or bakery foods are included in the official sampling plan for food safety. Only food products lacking any mention on the label of ingredients listed as declarable allergens by European Union regulations were collected. In particular, no allergens were listed among the ingredients, nor did the products carry precautionary labeling ("may contain"), indicating potential contamination by allergenic compounds. The samples were kept at their storage temperature until delivery to the Food Safety Control Laboratory. For the purposes of this study, samples collected during the investigation of cases of foodborne illnesses in which symptoms of allergic reactions were reported and in response to

reports of customer complaints were also included. In general, all samples coming from routine monitoring were analyzed within 10 days of collection; samples collected during foodborne illness investigations were analyzed the day of collection or the following day.

Methods. The laboratory methods were validated and accredited by the Italian Accreditation Body according to ISO 17025 (9). All methods showed a recovery between 65 and 113%; the relative standard deviation was <12%. Beta-lactoglobulin was detected by using the ELISA Ridascreen Fast Beta-Lactoglobulin Kit (R-Biopharm AG, Darmstadt, Germany). The kit is a sandwich enzyme immunoassay for the quantitative analysis of milk proteins in foods (e.g., sausages, ice cream, and bakery products), which may contain whey, milk, or milk powder proteins. The wells of strips are coated with specific antibodies against beta-lactoglobulin; when standard or sample solutions are added to the wells, beta-lactoglobulin binds to the specific antibodies, producing an antibody-antigen complex. The limits of detection and quantification are 0.2 and 0.5 mg/kg of beta-lactoglobulin, respectively. The upper limit of quantification is 13.5 mg/kg of beta-lactoglobulin.

Egg allergens were detected by using the ELISA Ridascreen Fast EI/Egg Protein Kit (R-Biopharm AG). The kit is a sandwich enzyme immunoassay for the quantitative analysis of whole egg proteins in foods (e.g., sausages, ice cream, pasta, and bakery products). The limits of detection and quantification are 0.27 and 0.55 mg/kg of whole egg powder, respectively. The upper limit of quantification is 13.5 mg/kg.

RESULTS

Table 1 presents the number of samples received and analyzed: 1,328 samples (654 for egg protein and 674 for beta-lactoglobulin screening) came from routine monitoring; 9 (6 for egg protein and 3 for beta-lactoglobulin screening) during foodborne illness investigations in response to a reported allergic reaction; and 229 in response to consumer complaints. Of this total of 1,566 samples, about one-half ($n = 775$) were screened for egg proteins; the breakdown by

TABLE 2. *Samples testing positive for egg proteins^a*

Year	Food matrix	DW ^b egg (ppm)	DW egg (mg/g)	Portion (g)	Ingested DW egg (mg)	Ingested DW egg proteins (mg)	ED01 egg proteins	×ED01
2010	Meatball	>13.5	>0.0135	100	>1.35	>0.648	0.03	>21.60
	Biscuit	11.53	0.01153	30	0.3459	0.166	0.03	5.53
	Biscuit	11.44	0.01144	30	0.3432	0.165	0.03	5.49
	Meat preparation	1.14	0.00114	100	0.114	0.055	0.03	1.82
	Fresh sausage	9.35	0.00935	100	0.935	0.449	0.03	14.96
	Hamburger	4.62	0.00462	100	0.462	0.222	0.03	7.39
	Fresh sausage	0.833	0.000833	100	0.0833	0.040	0.03	1.33
2011	Würstel	1.23	0.00123	50	0.0615	0.030	0.03	1
	Bovine meat	3.16	0.00316	100	0.316	0.152	0.03	5.06
	Hamburger	1.65	0.00165	100	0.165	0.079	0.03	2.64
	Meat preparation	>13.5	>0.0135	100	>1.35	>0.648	0.03	>21.60
	Fresh sausage	1.62	0.00162	100	0.162	0.078	0.03	2.59
	Würstel	>13.5	>0.0135	50	>0.675	>0.324	0.03	>10.80
	Hamburger	5	0.005	100	0.5	0.240	0.03	8.00
2012	Poultry meat	>13.5	>0.0135	100	>1.35	>0.648	0.03	>21.60
	Minced meat	3.64	0.00364	180	0.6552	0.314	0.03	10.48
	Minced meat	1.27	0.00127	100	0.127	0.061	0.03	2.03
	Fresh sausage	6.16	0.00616	100	0.616	0.296	0.03	9.86
	Fish product	5.37	0.00537	100	0.537	0.258	0.03	8.59
	Biscuits	0.7	0.0007	30	0.021	0.010	0.03	0.34
	Fish product	6.7	0.0067	100	0.67	0.322	0.03	10.72
2013	Fish product	1.01	0.00101	100	0.101	0.048	0.03	1.62
	Bakery product	2.8	0.0028	50	0.14	0.067	0.03	2.24
	Potato dumpling	3.97	0.00397	150	0.5955	0.286	0.03	9.53
	Fresh sausage	2.26	0.00226	100	0.226	0.108	0.03	3.62
	Pasta	>13.5	>0.0135	80	>1.08	>0.518	0.03	>17.28
	Hamburger	3.62	0.00362	100	0.362	0.174	0.03	5.79
	Cooked meat	0.91	0.00091	80	0.0728	0.035	0.03	1.16

^a Target allergenic concentrations compared with the ED01 based on the average reference levels of intake of food potentially ingested by a consumer. ED, eliciting dose.

^b DW, dried whole.

year was 249 samples in 2010, 183 in 2011, 155 in 2012, 84 in 2013, and 104 in 2014. The other half ($n = 791$) were screened for beta-lactoglobulin; the breakdown by year was 249 samples in 2010, 155 in 2011, 184 in 2012, 103 in 2013, and 100 in 2014. The number of samples collected each year was higher than planned: the final deviation is +84%. The four categories of food matrices were meat products (70%), bakery products (10%), infant food (3%), and other mixed products (17%).

The last column in Table 1 reports the number of samples that tested positive for the target allergen and for each year of the sampling period. Egg proteins were detected in 28 (3.6%) of 775 samples: 7 (2.8%) of 249 in 2010; 8 (4.4%) of 183 in 2011; 8 (5.2%) of 155 in 2012; 3 (3.6%) of 84 in 2013; and 2 (1.9%) of 104 in 2014. Beta-lactoglobulin was detected in 16 (2%) of 791 samples: 1 (0.4%) of 249 in 2010; 7 (4.5%) of 155 in 2011; 8 (4.3%) of 184 in 2012; and none in 2013 or 2014. In none of these cases did the food label mention either the presence of the target allergen among the ingredients or as a possible contaminant.

The majority of positive samples (40 of 44 samples) were collected through the PRISA program. The four other samples that tested positive were the following: one sample of infant food collected in 2012, following the report of an allergic reaction in a child that tested positive to milk

proteins, and three samples (two bovine minced meat samples and one fresh sausage sample) collected in 2012 in response to consumer complaints that tested positive for egg proteins.

Tables 2 and 3 report the distribution of positive samples and target allergen concentrations by food matrix category for egg proteins and beta-lactoglobulin, respectively. Nineteen (67.9%) of 28 samples in the meat products category, 4 (14.3%) of 28 bakery products, 3 (10.7%) of 28 fish products, and 2 (7.1%) of 28 pasta or dumpling products tested positive for egg proteins; 12 (75%) of 16 samples in the meat products category, 3 (19%) of 16 in infant food, and 1 (6%) of 16 bakery products tested positive for beta-lactoglobulin. For the level of positivity for beta-lactoglobulin, 7 (43.8%) of 16 samples were above the test upper limit of quantification of 13.5 mg/kg, with 5 (71.4%) of 7 in meat products and 2 (28.6%) of 6 in the infant food category, whereas for egg proteins, 5 (17.9%) of 28 samples were above the limit of 13.5 mg/kg, with 4 (80%) of 5 in meat products and 1 (20%) of 5 in the pasta category.

Taking the quantitative results of the analysis and the target allergen concentration expressed in parts per million (milligram per kilogram), we estimated the potential risk of allergen exposure for egg- and milk-allergic consumers. Tables 2 and 3 (columns 4 to 8) report the amount of

TABLE 3. Samples testing positive for beta-lactoglobulin^a

Year	Food matrix	β-lact (ppm)	β-lact (mg/g)	Portion (g)	Ingested β-lact (mg)	Ingested milk proteins (mg)	ED01 milk proteins	×ED01
2010	Würstel	2.3	0.0023	50	0.115	1.15	0.1	11.5
2011	Biscuit	9.05	0.00905	30	0.4525	4.525	0.1	45.25
	Infant food	3.78	0.00378	40	0.1512	1.512	0.1	15.12
	Hamburger	>13.5	>0.0135	100	>1.35	>13.5	0.1	>135
	Hamburger	7.66	0.00766	100	0.766	7.66	0.1	76.6
	Meatball	>13.5	>0.0135	100	>1.35	>13.5	0.1	>135
	Hamburger	1.77	0.00177	100	0.177	1.77	0.1	17.7
	Infant food	>13.5	>0.0135	40	>0.54	>5.4	0.1	>54
2012	Salami	>13.5	>0.0135	50	>0.675	>6.75	0.1	>67.5
	Salami	>13.5	>0.0135	50	>0.675	>6.75	0.1	>67.5
	Salami	11.95	0.01195	50	0.5975	5.975	0.1	59.75
	Sausage	12.54	0.01254	100	1.254	12.54	0.1	125.4
	Salami	>13.5	>0.0135	50	>0.675	>6.75	0.1	>67.5
	Salami	13.14	0.01314	50	0.657	6.57	0.1	65.7
	Meatball	1.39	0.00139	100	0.139	1.39	0.1	13.9
	Infant food	>13.5	>0.0135	40	>0.54	>5.4	0.1	>54

^a Target allergen concentrations converted into total milk protein content and compared with the ED01 based on the average for reference levels of intake of food potentially ingested by a consumer. β-lact, beta-lactoglobulin; ED, eliciting dose.

potentially eaten allergen according to the reference levels of intake of nutrients and energy (14). These amounts (grams of portion and milligrams of target allergens) were then compared with the eliciting dose (ED01) (1, 15), which represents the amount of allergenic proteins being able to protect 99% of the allergic population from objective symptoms of allergy, according to some authors. The range of target protein concentration in the positive samples was up to more than 100 times higher than the ED01.

DISCUSSION

The reduction in the planned number of samples collected through the Regional Integrated Plan for Food Safety roughly follows the decrease in the number of positive samples in the various food categories recorded the previous year. Each year, however, there were notable discrepancies between the number of samples planned for collection and those actually collected, which was much higher than expected. No reliable estimates can be made for samples collected outside of routine monitoring because it is impossible to predict the number of samples coming from cases of consumer complaints or foodborne illness investigations.

Though no statistically significant downward trend for positive samples could be determined, the number of noncompliant samples began to fall in 2013. This may be related to more familiarity and confidence of food producers with European Union food labeling regulations, which were published in 2011 but went into effect only 38 months later in December 2014.

Quantification of the two hidden food allergens we investigated is not required by law. To date, no limit of detection for milk and egg proteins has been established by European Union food safety regulations, which is why, during official control analyses for allergen detection, the extracts are not diluted. Instead, a simple positive result is sufficient to consider the sample as being uncompliant. Nonetheless, when allergen contamination in food is

detected during official controls or self-check procedures by food business operators, determining the levels of allergenic proteins can help identify the source of contamination in a food production process or facility. Moreover, recent experimental studies have attempted to establish an ED: the ED_x (e.g., ED5) is the dose at which *x*% of the allergic population would be expected to react with objective symptoms (1, 15).

Our results for egg contamination show that 5 of 28 samples were contaminated with egg proteins at a concentration higher than the upper limit of quantification (13.5 mg/kg); similarly, 7 of 16 samples tested positive for milk allergens, at a concentration higher than the upper limit of quantification (13.5 mg/kg). This suggests that though milk contamination is less frequent in the food products we tested, it is more relevant owing to the concentration of the allergenic target. Furthermore, 3 of the 16 samples testing positive for milk allergens came from infant foods, which raises cause for concern, as even a very small amount of allergen can induce an adverse reaction in children and infants.

Also, to evaluate the risk of exposure for consumers, we converted the concentrations of hidden allergens detected in the samples into the amount of food potentially ingested by a consumer and compared these values with the ED01. The portion sizes were defined according to the National Institute for Nutrition guidelines (14). The Voluntary Incidental Trace Allergen Labelling (VITAL 2.0, Allergen Bureau, Hobart Tasmania, Australia) tool sets the ED01 for egg at 0.03 mg of ingested total egg proteins and the ED01 for milk at 0.1 mg of ingested cow's milk proteins. A reference dose based on ED01 would, therefore, be expected to protect at least 99% of people allergic to the particular food from any objective reaction and minimize the probability of a serious reaction occurring in the remaining 1%.

The ED is not currently a legal limit nor a universally recognized threshold value that could reduce the health risk

to sensitized consumers or help food business operators design their hazard analysis critical control point protocols and plans; nonetheless, a comparison of the allergen amounts potentially ingested in a meal through positive samples can yield a better understanding of the risk for people with food allergies.

Furthermore, note that the actual consumption levels might be significantly higher than the standard servings established by government agencies. This difference needs to be taken into account, for example, if legal threshold values are set by European legislation on the provision of food information to consumers.

Regarding egg protein detection, it is important to highlight that proteins represent 48% of the National Institute of Standards and Technology powder used by the ELISA test used in this study to build the standard curve for quantification of samples: our estimates of egg proteins in the food samples indicate that in 26 of 28 cases, allergen exposure after eating even a single portion of food was higher than the ED01. To illustrate, the amount of egg proteins potentially ingested by a consumer in a single portion of a *würstel* (hot dog) sample (50 g) was equal to the ED01; a triple portion of a biscuit sample seemed to exceed the ED01.

Beta-lactoglobulin represents 10% of the total milk protein fraction on average, the total milk protein was 63 times (11 to 135) the ED01. Finally, the infant food samples were found to contain 15 and 54 times the ED01 of milk proteins.

Testing samples for the detection of beta-lactoglobulin only partially protects consumer health, as other milk derivatives, such as caseins and casein-based ingredients, are not detected even if present and potentially allergenic in food. For this reason, according to our findings and to better guarantee consumer safety, we suggested that the regional competent authority add in the Official Sampling Plan for Food Safety other target “milk proteins” to be detected in parallel with target “beta-lactoglobulin.” Indeed, starting in 2016, the analysis for official food sample testing now includes caseins as food allergens.

In conclusion, the total number of positive samples in this 5-year survey was about 3%: its relevance for public health cannot be regarded with indifference, also considering the high variability of the physiological response to food allergens. The presence of undeclared allergens in foods poses a potential health risk for sensitized consumers. Food business operators are responsible for the accurate indication on the food label of the presence of these ingredients or the potential contamination with allergens. The food industry will need to improve allergen control in their production plans and use labeling alerts only when stringent allergen control cannot be attained at a reasonable cost.

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