



Evaluation of rice as unregulated hidden allergen by fast real-time PCR

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1. Introduction

Rice is one of the most food crops consumed in the worldwide representing a staple food especially in Asian countries. Cases of IgE-mediated hypersensitivity of rice as inhalant, contact or food allergen have been reported mainly in Japan and Korea (Jeon et al., 2011; Togashi et al., 2019), whereas it is considered rare in western countries and indeed only few cases are published (Trcka et al., 2012; Pastorello et al., 2013; Tuckova et al., 2014).

Over 150 foods have been reported to cause allergic reactions among which rice is not considered at moment a priority allergen and it is not present in International Regulatory Chart (Gendel, 2012; FARPP, Food Allergy Research and Resource Program, <http://farpp.unl.edu/IRChart>) and in Annex II of Regulation (EU) No 1169/2011, as a substance subjected to mandatory labelling. Food allergen labelling concerns foods or ingredients responsible sketchily for the 90% of food allergic reactions (Gendel, 2012) also named "common foods" or "major allergens" that cause generally IgE-mediated food allergies (FA) and a large amount of publications reports on the prevalence of these allergens. In 2014, indeed EFSA reported a scientific opinion on the evaluation of allergenic foods and food ingredients including cereals containing gluten, milk and dairy products, eggs, nuts, peanuts, soy, fish, crustaceans, molluscs, celery, lupin, sesame, mustard and sulphites. Moreover, most of the data of studies resulting from the EU-funded multidisciplinary Integrated Project EuroPrevall were conducted on 14 inhalant allergens, latex and 24 foods (milk, egg, fish, shrimp, peanut, hazelnut, peach, apple, celery, soy, walnut, sesame seed, mustard seed, kiwi, wheat, sunflower seed, carrot, buckwheat, banana, lentil, tomato, poppy seed, corn, melon) (Fernandez-Rivas et al., 2015). Additional studies on epidemiology of

"common food" were provided in a recent review of Sicherer and Samson (2018). The methodology generally adopted in these studies and reported in the reviews, included medical history and determination of the sensitization measure by serum-specific IgE (sIgE) and/or skin prick test (SPT) to "major food allergens". Only positive results are followed, when possible, by oral food challenges (OFC) to confirm the diagnosis of food allergy and, in some few cases, a double-blind placebo-controlled food challenge (DBPCFC), considered the gold standard (Muraro et al., 2014), was performed.

The rice, as allergen, became more relevant and present in studies related to non-IgE mediated food allergy, especially to food-protein induced enterocolitis syndrome (FPIES). FPIES is one of most prevalent non-IgE FA besides (o next to) eosinophilic esophagitis (EoE), proctocolitis (FPIAS) and enteropathy (FPE). FPIES, characterized by acute, chronic and atypical forms, notably in infancy, lacks of the typical expected IgE-mediated symptoms, indeed FPIES has negative SPT and sIgE to the trigger food, only in the atypical form specific IgE are present (Lonzano-Ojalvo et al., 2016; Bingemann et al., 2018). Moreover, FPIES can co-exist with other FPIES and/or IgE mediated food allergies (Nowak-Węgrzyn et al., 2017). At present, the diagnosis of FPIES is often based on symptoms, clinical history, diet for elimination and specifically OFC, considered the gold standard (Muraro et al., 2014; Nowak-Węgrzyn et al., 2017; Bingemann et al., 2018). Internationally, the most common FPIES trigger food is cow milk (CM), followed by soy, but among solid food, rice is the most commonly reported and the more common in Australia (Mehr et al., 2017; Bingemann et al., 2018). The prevalence varies by country and actually is unknown (Nowak-Węgrzyn et al., 2017; Bingemann et al., 2018). Currently the rice is considered as a safe food and an hypoallergenic foodstuff and then it is included in

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Table 1

Description of primers and probes.

System Name	Name	Sequence (5'-3')	Amplicon length (bp)	Reference
PLD-GR	PLD-GR For	GGCAAGAACAACACCAATTGACA	124	QT-TAX-OS-002 ^a
	PLD-GR Rev	CGGCTTTCCATGCAAATGA		
	PLD-GR Probe	FAM- ATTCGCGCGCCAGAACTTCA -TAMRA		
Act	ACTIN-F	CAAGCAGCATGAAGATCAAGGT	103	Laube et al. (2010)
	ACTIN-R	CACATCTGTTGGAAAGTGCTGAG		
	ACTIN-TMP	FAM- CCTCCAATCCAGACACTGTACTTCTCTC -TAMRA		

PLD-GR: phospholipase D $\alpha 2$ gene; Act: actin gene; bp: base pairs; FAM: 6-carboxyfluorescein; TAMRA: tetramethylrhodamine quencher dye.

^a <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>.

diets for allergic people and used in weaning food addressed to infancy.

In the last years, the rice demand in the European Union is growing significantly as a mirror of changing society related to increasing of coeliac patients; attention to healthiness, considering rice as dietary food; aging of the population and coming of crisis pushing to meet the food needs at a lower cost; migration flows of rice consumer ethnic groups. Therefore, food allergens that are not reported to be common or that are not known as allergic in a country can be an allergic food in other countries, depending on geography, dietary habits, food preparation and age at which food is first consumed (Lucas et al., 2004; Verhoeckx et al., 2016).

To understand the categorisation and prioritisation of allergic food according to public health importance, there is a need of prevalence and potency data, scarcely available for rice (Houben et al., 2016).

In this paper we report a fast real-time PCR based method to detect the presence of rice, not listed in labelling among the ingredients, in a commercial multigrain cereal cream baby food for weaning investigated as a cause of serious allergic reactions in a young people allergic to rice, hospitalised in Umbria (central Italy) in 2018, after ingestion of the multigrain cereal cream. No immunological commercial kits were available for this purpose.

Moreover, recent studies supply information on the presence of undeclared “major allergens” in foods (Pierboni et al., 2018; Blom et al., 2018), while nothing is known about the presence of minor allergen as rice.

Therefore, although the presence of rice as an allergenic ingredient is not required to be stated in label by the current legislation, considering the geographical and socio-economic variations, the emerging non-IgE mediated allergies and the only presence of snapshots on allergy problems, it seemed to be useful to investigate on the presence and quantity of undeclared rice in some commercial food samples.

2. Material and methods

2.1. Samples

Two commercial packs of the multigrain cream for weaning involved in the infant rice allergy case, with different batch number (named 01 and 02), were analysed to verify the presence of rice. The label of the multigrain cream listed whole oat, wheat, barley, spelled, rye flour, wheat semolina and B1 vitamin as ingredients, while rice was not stated.

Afterwards, thirty-one food products without rice declared in labelling, collected between January 2018 and February 2019 in supermarkets of Umbria and Marche regions were analysed for rice detection. The collected samples were received as a control of national plan GMO (genetically modified organism). The labels stated the presence of soybean in 9 samples, maize in 14 samples, wheat in 3 samples, while the rest of the samples contained more than one plant species. When necessary, the whole samples were milled with Grindomix GM200 (Retsch, Haan, Germany) and homogenized with plastic bag technique (EUR 27021 EN, 2014).

The certified reference materials (CRM) used in the in-house validation of the rice method (QT-TAX-OS-002, <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>) were purchased from AOCS (American Oil

Chemists' Society, <https://www.aocs.org/crm>) or IRMM (Institute for Reference Materials and Measurements, https://ec.europa.eu/jrc/sites/jrcsh/files/rm_catalogue_190320.pdf/) listed in the notes of Supplementary Table 1.

2.2. DNA extraction

The first step of analysis was a duplicate DNA extraction of each sample, performed following the protocol selected and validated by Pierboni et al. (2018). In this DNA extraction method, the Annex A.3 of ISO 21571:2005/Amd.1:2013 protocol has been followed until CTAB-precipitation step, and combined with the commercial kit for DNA purification NucleoSpin® gDNA Clean-up (Macherey-Nagel), according to the manufacturer instructions, instead of DNA precipitation step (isopropanol and ethanol 70%). Test portions of 2 g of the commercial samples and 200 mg of the certified reference materials were extracted. Sterile distilled water instead of sample was used as negative extraction control (ISO 24276:2006/Amd.1:2013). To increase the amount of extracted DNA from batches of multigrain cream for weaning, two aliquots of the same sample instead of one, were added to the same column of purification. The quantity of extracted nucleic acids was determined fluorimetrically using a BioSpectrometer® fluorescence (Eppendorf®, Hamburg, Germany) and a Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.3. Fast real-time PCR

Real-time PCRs were run in fast mode by 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). Each reaction was performed in a reaction volume of 20 μ L with 1X of TaqMan® Fast Universal PCR Master Mix (2X) No AmpErase UNG (Thermo Fisher Scientific), 2 μ L of DNA and primers/probes (Table 1) synthesized by Invitrogen (Thermo Fisher Scientific), used at concentrations reported in Torricelli et al. (2016) and in Supplementary Table 2, for *actin* and *phospholipase D $\alpha 2$* (PLD-GR) (QT-TAX-OS-002, <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>) genes, respectively.

The real-time PCR was carry out using the following cycling conditions: 95 °C for 20 s, 40 cycles with 95 °C for 3 s and 60 °C for 30 s. Data were analysed by a SDS 2.4 software (Thermo Fisher Scientific, 2012).

Each PCR run included no template control (NTC) and a positive amplification control.

2.4. In-house validation of rice method

First of all, the optimization of primer and probe concentrations was performed, testing three different concentration of primers (150 nM, 300 nM, 900 nM), fourfold each of nine combinations. Four concentrations of probe (100 nM, 150 nM, 200 nM, 250 nM) were tested with selected optimal combination of primers, always fourfold for each concentration. The test was conducted on 120 haploid genome size (hge) considering a mass of haploid genome size (1C) of 0.5 pg in rice specie (Kew Gardens C-value database, <https://cvalues.science.kew.org/>), computing the number of genome equivalents per μ L on the basis of the mass in pg, that is in this case 100 ng/ μ L multiplied 1000 divided 0.5 pg,

resulting in 200,000 hge. To identify the optimal concentration of PLD-GR primers, the first parameter considered was ΔRn (pre-PCR minus post-PCR datapoints of normalized intensity of the reporter dye), followed by Cq and SDr (repeatability standard deviation). Probe concentration was chosen considering the lowest mean quantification cycle (Cq) as an indicator for the best sensitivity, together with the lowest repeatability standard deviation (SDr) as indicator of repeatability.

The performance of qualitative and quantitative real-time PCR method was tested following the parameters and acceptance criteria recommended in guidelines (EURL-EUR 29015 EN, 2017; EURL-JRC95544, 2015; Broeders et al., 2014).

The in silico and experimental specificity was assessed by database BLAST (Basic Local Alignment Search Tool. <http://blast.ncbi.nlm.nih.gov/Blast.cgi/>) and testing in duplicate 53 test materials, listed in Supplementary Table 1.

The absolute LOD (limit of detection) was evaluated on nine serial dilutions each in ten replicates (LOD₁₀): 320 hge/0.16 ng; 160 hge/0.08 ng; 80 hge/0.04 ng; 40 hge/0.02 ng; 20 hge/0.01 ng; 10 hge/0.005 ng; 5 hge/0.0025 ng; 3 hge/0.00125 ng; 1 hge/0.000625 ng. The LOD₉₅ or LOD of method with a 95% confidence level, was verified with 60 amplifications, starting from last dilution with 10/10 positive signals. The data obtained from LOD of method were also used to evaluate the cut-off of PLD-GR system (Accredia DT-07-DL/DS, 2013). The linear regression analysis on the first five dilutions of LOD₁₀, had provided a slope and a correlation coefficient R^2 useful to evaluate respectively the efficiency and the linearity of qualitative method.

The robustness of qualitative and quantitative method consisted in a multi-factorial experiment performed in triplicate. Two PCR runs were carried out on different thermal cyclers, 7500 and 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific), with a different Master mix concentration (−10%), different primer and probe concentrations (−30%), different reaction volumes (+1 μ L and −1 μ L) and with an annealing temperature farther than T_m (melting temperature) of primers (Pierboni et al., 2016). The target amount was three times the LOD for qualitative method while for the quantitative one it was represented by two preparations of 0.1% and two of 1% (EURL-JRC95544, 2015).

The absolute LOQ (limit of quantification) was evaluated where RSDr was below 25% (relative repeatability standard deviation) determined from the LOD₁₀ data.

Linearity, efficiency, trueness and precision (RSDr) of the quantitative method, were assessed from four results of two reference samples at different concentrations, 1% and 0.1% w/w of rice, each concentration obtained from two preparation and each performed in quadruplicate in two different PCR plates under repeatability conditions. The different concentrations of rice or reference samples (RS) were obtained diluting rice flour DNA with non-modified soybean and maize DNA (EURL-EUR 29015 EN, 2017).

2.5. Analysis of food samples

The quality of DNA was verified by an inhibition test (Waiblinger and Grohmann, 2014; EURL-EUR 29015 EN, 2017) using real-time PCR assay targeting the *actin* gene (Laube et al., 2010; Torricelli et al., 2016). The test consisted in analysing two real-time PCR replicates of undiluted DNA and its dilution 1:4 for each extracted sample. In absence of inhibitors, the difference between the measured mean cycle quantification (Cq) of undiluted and diluted DNA (ΔCq) should be of 2, with an acceptability range of 1.5–2.5.

The presence of rice was tested using a real-time PCR assay targeting the *phospholipase D α 2* gene (PLD-GR) (QT-TAX-OS-002, <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>). The qualitative and quantitative real-time PCR (not inhibition test) were performed in single and triplicate for each DNA extracted from sample, respectively.

The dynamic range from 80,000 hge to 40 hge was based on the standard curve of five concentration levels (40, 4, 0.4, 0.04, 0.02 ng),

each in triplicate starting from the same DNA rice used for method validation. qPCR run included no template control (NTC) and one positive amplification control.

3. Results and discussion

3.1. Performances of qualitative and quantitative PLD-GR method by fast real-time PCR

The in-house validation of qualitative and quantitative real-time PCR is carried out to verify the performance of rice method in fast mode, because the different cycling conditions with respect to the original method (QT-TAX-OS-002, <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>) may affect performances such as efficiency and specificity. Then, the parameters verified in qualitative real-time PCR were: specificity, LOD, robustness, efficiency, linearity. The parameters verified in quantitative real-time PCR were: LOQ, trueness, precision, linearity, efficiency and robustness (EURL-EUR 29015 EN, 2017; EURL-JRC 95544, 2015; Broeders et al., 2014).

The DNAs used in-house validation were tested previously with inhibition test, resulting suitable for intended use.

The combination with the highest ΔRn was 900 nM/900 nM (forward/reverse), and the 300 nM/900 nM showed a high ΔRn , close to the other one, but with better mean Cq and SDr, and it was then selected as optimal combination of forward and reverse primers (Supplementary Table 2). This combination was tested with increase of probe concentrations providing the better mean Cq, a good SDr and a high ΔRn , with 250 nM of probe (Supplementary Table 2). The 300 nM/900 nM (forward/reverse primer) and 250 nM (probe) concentrations were selected for PLD-GR method in fast real time PCR.

The in silico specificity confirmed an identity of 100% for *Oriza sativa*, 98% for *Oriza sativa* accession number AB001919.1, 93% for *Oriza branchyantha*, 90% for *Hordeum vulgare* (barley) and lower identity (below 90%) and query cover (below 86%), for *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), *Triticum aestivum* (wheat) and *Solanum pennelli* (wild tomato). The experimental specificity provided positive results only for rice samples, with no false positive or false negative results confirming the specificity of PLD-GR system for rice detection in fast real-time PCR (Supplementary Table 1).

The LOD₁₀ test resulted in 10/10 positive signals for dilutions of 3 hge/0.00125 ng, not confirmed by the LOD₉₅ (Supplementary Tables 3 and 4). Therefore, the upstream dilutions were tested and 59 amplifications out of 60 resulted in absolute LOD of 10 hge/0.005 ng, with a practical LOD of 0.005%, considering mass of haploid genome of 0.5 pg in rice specie, corresponding to 200,000 hge/100 ng. The 60% of negative results obtained for 1 hge, attested the correctness of serial dilutions (Supplementary Table 3). The amplification efficiency and linearity of qualitative PCR analysis were attested on 89% and 0.99% respectively, according to the acceptance criteria (Broeders et al., 2014). The robustness of qualitative PCR showed a total concordance (Supplementary Table 5), confirming the method capacity to remain unaffected by small deviations (EURL-JRC95544, 2015). The LOQ value, identified with the RSDr $\leq$ 25%, was 40 hge/0.02 ng, corresponding to practical LOQ of 0.02% considering mass of haploid genome of 0.5 pg in rice specie, corresponding to 200,000 hge/100 ng (Kew Gardens C-value database, <https://cvalues.science.kew.org/>) (Supplementary Table 3).

The performances of quantitative PLD-GR method in fast real-time PCR meet the acceptance parameters as shown in Supplementary Table 6, satisfying also the multifactorial robustness test with RSDr estimated below 30% (Supplementary Table 7).

3.2. Results of the food samples

The quantity of food sample DNAs extracted varied from a minimum of about 10 ng/ μ L in soybean drink to a maximum of about 200 ng/ μ L in semolina. Anyway, DNAs were tested by actin system in fast real time

Table 2

Results by qualitative real-time PCR of weaning food suspected culprit of allergenic reaction in the child.

ID samples	real-time PCR		
	actin		PLD-GR
	mean Cq	Δ Cq	
A-01	21.72	1.86	36.43
B-01	21.94	1.90	36.95
A-02	21.67	2.00	37.21
B-02	21.81	2.02	36.14

A and B: two DNA extracts from the same sample; 01 and 02: two different batches.

Δ Cq: difference between mean Cq of undiluted DNA and its dilution 1:4.

PLD-GR: phospholipase D α 2 gene.

Cq: cycle quantification.

PCR, with a maximum of 200 ng in reaction, provided Cq from about 21 to 26 and the Δ Cqs, within the acceptable range and therefore without inhibitors (Tables 2 and 3).

Then, all DNAs were suitable to be analysed with the validated PLD-GR method.

The presence of rice was found in both batches of weaning food, as shown in Table 2. The samples were detected with high Cq, below the cut-off estimated for the method PLD-GR, of 37.59 Cq, then suggesting a low amount of target, probably due to a cross-contamination phenomenon in the production chain. The presence of rice was revealed using at

least 100 ng of genomic DNA in the reaction, since the quantity in ng corresponds to the DNA of the different species present in the sample and not only of the DNA target. Therefore, a good quality and quantity of DNA is important for the sensitivity and reliability of the PCR method.

Following the positive results, obtained in the involved weaning food, 31 food samples not declaring rice in the label were tested (Table 3). The samples from 1 to 9, in which soybean was stated in the label, resulted negative to the presence of rice (Table 3). The presence of undeclared rice resulted in five of the 31 samples, corresponding to a positivity of about 16% (Table 3). Considering the multigrain cream for weaning (A-01_A-02 and B-01_B-02, the same sample with different batch) the positivity rises to 19%. Three of the five positive samples were identify in flour maize or polenta, then associated to maize ingredient, another was semolina, containing wheat as the main ingredient, and the last was identified as a cereal-soup. Therefore, it seems that the presence of rice is associated mainly to cereals. The high obtained Cqs, ranging from 30 to 35, indicate probably accidental contaminations in the production chain. In all qualitative and quantitative real-time PCR runs, negative extraction and amplification controls resulted negative, while positive amplification controls were positive.

The quantitative real-time PCR was performed to better understand the quantity of rice in the samples. The calibration curve showed, according to EURL-JRC95544, 2015 parameters, an efficiency of about 94% and a linearity of 1 (Supplementary Table 8). In Table 4 are shown the results in Cqs and practical ng and hge for each extract of each sample extrapolated from the equation of the calibration curve with

Table 3

Qualitative results of market samples in real-time PCR for rice presence.

ID	market samples	plant species	real-time PCR actin				real-time PCR
			mean Cq		Δ Cq		PLD-GR
			1°extract	2°extract	1°extract	2°extract	
	type	declared in the label					mean Cq $\pm$ SDr
1	soya drink	soya	24.27	24.37	2.13	2.03	undet
2	soya drink	soya	24.99	24.98	2.03	1.95	undet
3	soya drink	soya	23.28	23.28	2.04	1.9	undet
4	soya drink	soya	23.61	23.62	1.96	1.96	undet
5	soya drink	soya	23.53	23.9	2.25	2.08	undet
6	soya flour	soya	23.58	23.41	1.89	1.97	undet
7	whole soya flour	soya	21.06	21.15	2.02	1.92	undet
8	yellow soya	soya	20.87	20.88	2.10	2.13	undet
9	soya hamburger	soya	23.27	23.19	1.85	1.84	undet
10	mix flour	maize and others ^a	24.25	23.88	2.08	1.88	undet
11	mix flour	maize and others ^a	23.19	23.44	1.85	2.07	undet
12	pasta gluten free	maize	23.27	23.47	2.09	2.27	undet
13	maize flour	maize	24.27	24.17	2.27	1.77	33.98 $\pm$ 0.30
14	polenta (corn meal)	maize	26.24	26.91	2.23	2.07	undet
15	polenta (corn meal)	maize	24.03	23.73	2.14	2.15	30.37 $\pm$ 0.25
16	polenta (corn meal)	maize	24.06	24.52	2.00	2.06	33.80 $\pm$ 0.74
17	polenta (corn meal)	maize	23.39	23.31	2.12	2.11	undet
18	polenta (corn meal)	maize	22.98	23.03	2.02	2.18	undet
19	polenta (corn meal)	maize	24.6	24.7	1.94	1.92	undet
20	polenta (corn meal)	maize	23.23	23.38	2.04	1.8	undet
21	polenta (corn meal)	maize	22.6	22.75	1.95	2.03	undet
22	polenta (corn meal)	maize	24.87	24.65	2.04	2.08	undet
23	polenta (corn meal)	maize	23.26	23.11	2.08	2.17	undet
24	polenta (corn meal)	maize	24.69	24.62	1.94	2.11	undet
25	polenta (corn meal)	maize	24.15	24.08	2.22	1.94	undet
26	biscuits for baby	wheat	25.86	26.05	2.26	2.22	undet
27	semolina babyfood	durum wheat	23.63	22.95	1.98	2.00	34.90 $\pm$ 0.48
28	cereal biscuits	whole wheat and others ^a	25.03	24.81	2.00	1.99	undet
29	crackers	wheat	25.86	26.03	1.73	1.72	undet
30	polenta with cheese	maize, onion	22.98	22.78	2.08	2.05	undet
31	5 cereal soup	barley and others ^a	25.02	24.9	2.18	2.16	34.61 $\pm$ 0.02

PLD-GR: phospholipase D α 2 gene.

Cq: cycle quantification.

Undet: undetermined.

Δ Cq: difference between mean Cq of undiluted DNA and its dilution 1:4.

SDr: standard deviation of repeatability.

^a Plant species declared in the label in samples: 10 (maize, barley, rye, flax, millet); 11 (maize, spelled, wheat, barley, broad beans, peas); 28 (whole wheat, oats, spelled, barley); 31 (barley, rye, wheat, oats, buckwheat, potato, onion, celeriac, bean, carrots, peas, tomato, leek, green beans, turnip, celery).

Table 4
Results of rice quantity in food samples positive to presence of PLD-GR gene.

ID	13 (maize flour)			13 ^a (maize flour)			15 (polenta)			15 ^a (polenta)			16 (polenta)			16 ^c (polenta)			27 (semolina)			27 ^b (semolina)			
	replicates	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng			
1		34.889	23.339	0.012	33.472	59.341	0.030	30.226	503.339	0.252	30.442	436.727	0.218	34.566	28.881	0.014	34.401	32.185	0.016	34.955	22.350	0.011	34.121	38.720	0.019
2		34.382	32.608	0.016	33.754	49.299	0.025	30.519	415.049	0.208	30.507	418.283	0.209	34.960	22.278	0.011	34.377	32.711	0.016	34.331	33.708	0.017	34.685	26.699	0.013
3		34.374	32.781	0.016	34.007	41.722	0.021	30.586	397.262	0.199	30.568	401.958	0.201	34.731	25.907	0.013	34.019	41.390	0.021	34.257	35.407	0.018	34.615	27.954	0.014
mean Cq		34.548	29.576	0.015	33.745	50.121	0.025	30.444	438.550	0.219	30.506	418.989	0.209	34.752	25.689	0.013	34.266	35.429	0.018	34.514	30.488	0.015	34.474	31.124	0.016
SDr		0.296	5.402	0.003	0.268	8.838	0.004	0.191	56.810	0.028	0.063	17.395	0.009	0.198	3.307	0.002	0.214	5.170	0.003	0.384	7.099	0.004	0.308	6.608	0.003
RSDr		0.855	18.265	0.015	0.793	17.634	0.004	0.628	12.954	0.207	0.415	4.152	0.009	0.570	12.873	0.002	0.624	14.592	0.003	1.111	23.285	0.004	0.893	21.230	0.003

ID	31 (cereal soup)			31 ^a (cereal soup)			A-01 (cereal cream)			B-01 (cereal cream)			A-02 (cereal cream)			B-02 (cereal cream)			soya-rice drink			soya-rice drink			
	replicates	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng	Cq	hge	ng			
1		33.951	43.286	0.022	34.607	28.116	0.014	36.524	7.953	0.004	36.073	10.702	0.005	38.238	2.571	0.001	37.050	5.624	0.003	31.824	175.752	0.088	32.150	141.779	0.071
2		34.016	41.472	0.021	34.604	28.155	0.014	36.973	5.918	0.003	36.722	6.979	0.003	37.122	5.364	0.003	36.723	6.978	0.003	32.330	125.959	0.063	32.273	130.785	0.065
3		34.073	39.948	0.020	34.165	37.606	0.019	36.470	8.241	0.004	36.179	9.984	0.005	37.782	3.473	0.002	37.027	5.712	0.003	32.264	131.515	0.066	31.929	163.987	0.082
mean Cq		34.014	41.568	0.021	34.459	31.292	0.016	36.656	7.371	0.004	36.325	9.221	0.005	37.714	3.803	0.002	36.933	6.105	0.003	32.139	144.409	0.072	32.117	145.517	0.073
SDr		0.061	1.671	0.001	0.254	5.467	0.003	0.276	1.266	0.001	0.348	1.975	0.001	0.561	1.425	0.001	0.183	0.757	0.0004	0.275	27.286	0.014	0.174	16.914	0.008
RSDr		0.179	4.020	0.020	0.738	17.472	0.020	0.753	17.181	0.020	0.959	21.420	0.020	1.488	37.480	0.020	0.494	12.406	0.020	0.856	18.895	0.020	11.623	11.623	0.020

hge: haploid genome equivalents.

SDr: standard deviation of repeatability.

PLD-GR: phospholipase D α 2 gene.

Cq: cycle quantification.

RSDr: relative repeatability standard deviation.

^a Second DNA extracted.

intercept of 28.13 for ng and 39.67 for hge (Supplementary Table 8). Only the samples 15 (polenta) and the positive amplification control (soya-rice drink) was quantifiable and with RSDr below 25%. The samples 13 (maize flour) and 31 (cereals soup) were doubt as only one of two the sample extracts was quantifiable while the other not, because the mean Cq was higher than the Cq LOQ of the method, corresponding to the last point of the calibration curve (Supplementary Table 8). The other samples (16 - polenta; 27 - semolina babyfood; A-01, B-01, A-02 and B-02 - multigrain cream for weaning) were below LOQ, then not quantifiable. The real-time PCR method allowed to detect rice DNA in samples and not the protein. No commercial immunologic methods are available up to now to evaluate the rice allergen presence and quantity.

4. Conclusions

The rice is consider a minor allergen, thus widely used in people diet. The availability of data on rice as allergen is limited and uncertain, due to the lack of registries and of recent studies mostly focused on regulated major allergens.

This study focusing attention on rice as allergen, looking for understanding the possible relationship between the allergic reactions of an Italian child allergic to rice and an unintentional presence of the allergen in a multigrain cream for weaning.

The molecular analysis carried out to detect the presence of rice resulted positive with a low amount probably due to cross-contamination phenomena occurred in the production chain. Therefore, the presence of rice may be the cause of the adverse reaction observed in the allergic child, although the limited data available does not allow to understand if this dose can trigger allergic reactions in sensitive patients.

Indeed, the study highlights the presence of non-ingredient rice in other food products even in low amounts. Therefore, it can be possible that the widespread use of rice as a substitute food in allergic diet and weaning in industrial preparations, contributes to accidental food contaminations and involuntary consumption.

Moreover, the geographical and socio-economic variations could influence the distribution of food allergies. This aspect could assume a vital importance, considering also emerging non-IgE mediated allergies such as FPIES, in which rice is becoming a relevant allergen also in geographical areas where it has not yet occurred, remembering the strict avoidance of trigger food in allergic people.

Besides, this work proposes a DNA-based analysis method by fast real-time PCR, a tool for reliable detection and then management of rice allergen throughout the food production chain and its distribution, in a short time. Since allergens may be present in relatively low amounts, there is a need for sensitive analytical methods such as the qualitative real-time PCR method described herein. Qualitative PCR offers when compared to quantitative real-time PCR a higher sensitivity, allowing the detection of trace allergens. Therefore, qualitative analysis is recommended for food safety control and prevention. At present, quantification is not required by legislation and the measured DNA cannot be related to the amount of the allergenic protein, but rather to the amount of the allergenic ingredient in food. Indeed, it would be important to define the minimum requirements that an analytical method for allergen detection must meet in order to be consider as reliable.

Finally, the establishment of functional and accessible registries and available databases would be essential to monitoring, steady update and evaluation of effective prevalence, and to the control, prevention and prediction of food allergies in order to raise awareness, reduce risks, costs and promote wellness.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcs.2020.102929>.

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