

A comprehensive molecular approach in fish allergy: Usefulness in daily clinical practice

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ARTICLE INFO

Keywords:

Fish allergy
Molecular diagnosis
β-parvalbumins
Aldolase
Enolase

ABSTRACT

Background and aims: Diagnosis of fish may represent an important challenge for the allergists. This study aimed to evaluate the diagnostic sensitivity of an *in vitro* multiplex assay using a comprehensive panel of fish allergens and the cross-reactivity patterns between different molecular components.

Methods: 56 subjects with fish allergy were enrolled. All patients underwent specific IgE measurement using the Allergy Explorer-Alex 2TM multiplex assay (Macroarray Diagnostics, Vienna, Austria)

Results: The single β-parvalbumins Clu h 1, Cyp c 1, Gad m 1, Sal s 1, Sco s 1, Thu a 1 and Xyp g 1 scored positive in 75.0%, 67.8%, 62.5%, 80.3%, 80.3%, 78.8% and 73.2% patients, respectively. 14.3% scored positive for the α-parvalbumin (Raj c-parvalbumin), and 16.1% for the aldolase + enolase (Gad m 2 + 3) components. 92.8% reacted to at least one β-parvalbumin and 96.4% to at least one of the allergens tested. Overall sensitivity was higher than that obtained using commercial extracts of cod, salmon and tuna for skin prick test (75.8%) and IgE detection (92.3%).

Conclusions: The Alex 2 showed high diagnostic sensitivity and it might be used as an additional assay to investigate the cross-reactivity patterns between different molecular components, looking for potentially safe fish species.

1. Introduction

Fish allergy is one of the most common causes of food allergy affecting about 0.1–0.4% of the world's population [1,2]. There is much biodiversity among fishes (more than 30.000 species have been described) and taxonomically they are divided into two main subgroups: Osteichthyes (bony fishes) and Chondrichthyes (cartilaginous fishes). Most edible fishes belong to the former group and the most frequently consumed species belong to a limited number of orders [3]. However,

there are considerable differences in the quantity and type of fish consumed worldwide. In Europe the highest fish consumption is found in Scandinavia, Spain and Portugal and cod and salmon represent the most widely consumed types [4]. Symptoms of a fish allergy can vary from isolated gastrointestinal symptoms, such as oral allergy syndrome, nausea, vomiting, urticaria-angioedema, respiratory involvement, and anaphylaxis. Most fish allergic patients have reactions to multiple fish species [5] due to the high cross-reactivity among the parvalbumins present in different fish species. Fish parvalbumin is a highly stable

Abbreviations: SPT, Skin prick test; PPT, Prick-prick test; IgE, Immunoglobulin E; CRD, Component Resolved Diagnosis.

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<https://doi.org/10.1016/j.cca.2022.06.010>

Received 1 May 2022; Received in revised form 15 May 2022; Accepted 7 June 2022

Available online 16 June 2022

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protein of low molecular weight (10–12 kDa), belongs to the EF-hand helix-loop-helix domain family of calcium-binding proteins, is involved in the regulation of the intracellular calcium concentration during muscle relaxation, and represents the most important fish allergen [6,7]. The high sequence identity among the parvalbumins contained in different species of fish is the molecular basis of the cross-reactivity. There are two distinct lineages of parvalbumin: β -parvalbumin and α -parvalbumin. The former, reported to be the major allergen, is predominant in muscle tissue of bony fish, while the latter is prevalent in cartilaginous fish [8]. Since amino acid sequence homologies between the two lineages of parvalbumin are usually < 50%, low cross-reactivity occurs between α - and β -parvalbumin. In 2013 Kuehn et al. [9] identified the 47–50 kDa enolase and the 40 kDa aldolase as important new fish allergens in cod, salmon and tuna, possibly relevant for the diagnosis of fish allergy, particularly in the absence of parvalbumin reactivity. Both enolase and aldolase show low thermal resistance and limited inter-species cross-reactivity [9]. Finally, other fish allergens such as tropomyosin, triosephosphate isomerase, collagen and pyruvate kinase have been described in some subjects, but their clinical relevance remains unclear [10–13].

The diagnostic work-up of fish allergy is based on the patient's history, skin prick testing (SPT), specific IgE determination and when indicated, an oral food challenge. Extracts of many types of fishes are available for sIgE determination, while for several years only the parvalbumin from cod (*Gadus callarias*- Gad c 1) and carp (*Cyprinus carpio*- Cyp c 1) were available for the component resolved diagnosis (CRD). Recently, a multiplex assay was able to detect specific IgE to the β -parvalbumins of herring (*Clupea harengus*, Clu h 1), carp (*Cyprinus carpio*, Cyp c 1), cod (*Gadus morhua*, Gad m 1), salmon (*Salmo salar*, Sal s 1), mackerel (*Scomber scombrus*, Sco s 1), tuna (*Thunnus albacares*, Thu a 1) and swordfish (*Xiphias gladius*, Xip g 1), to the α -parvalbumin of thornback ray (*Raja clavata*, Raj c parvalbumin), and β -enolase + aldolase of cod (Gad m 2 + 3) components are available in the market.

This study aimed to evaluate the molecular profile of a cohort of patients with fish allergy using the aforementioned multiplex assay and to assess its impact on the diagnostic workup of fish allergy.

2. Materials and methods

2.1. Study population

Six Italian allergy Centres took part in a multicenter cross-sectional observational clinical survey of fish allergy. In every participating centre, doctors consecutively enrolled during their routine activity patients with fish allergy, diagnosed between May 15th and December 30th, 2021. The patient population included 56 subjects, 34 males and 22 females, mean age of 24.9 years (range: 2–80; 11 patients were less than 16 years old). All patients experienced symptoms following the ingestion of fish, and in some cases even after contact and/or inhalation during cooking or processing. In all patients, the history of fish allergy was confirmed by a positive skin prick test (SPT) with commercial fish extracts of cod and/or salmon and/or tuna (Lofarma, Milan, Italy) and/or by a positive prick-prick test (PPT) with the culprit fish (either raw or cooked) and/or by the detection of specific IgE to cod, salmon and/or tuna extracts. SPT and PPT were regarded as positive when the wheal diameter was 3 mm larger than the negative control.

2.2. Specific IgE measurement

Specific IgE to cod, salmon and tuna extract were measured using the ImmunoCAP system (Thermo Fisher, Uppsala, Sweden) following the manufacturer's instructions; levels greater than 0.15 kUA/L were considered positive. In all the 56 enrolled subjects a deeper evaluation was carried out by a multiplex platform (Allergy Explorer-Alex 2TM, Macroarray Diagnostics, Vienna, Austria) evaluating a broad profile of fish allergens including β -parvalbumins from cod (*Gadus morhua*- Gad m

1), carp (*Cyprinus carpio*- Cyp c 1), herring (*Clupea arengus*- Clu h 1), salmon (*Salmo salar*- Sal s 1), mackerel (*Scomber scombrus*- Sco c 1), tuna (*Thunnus albacares*- Thu a 1) and swordfish (*Xiphias gladius*- Xip g 1), α -parvalbumin from thornback ray (*Raja clavata*- Raj c-parvalbumin), and aldolase + enolase from cod (Gad m 2 + 3). Following the manufacturer's instructions, IgE levels ≥ 0.3 kUA/L were considered positive.

Since the diagnosis of fish allergy was carried out within routine activity it was not confirmed by blinded or open oral food challenges due both to the severity of several reactions and because many centres were not sufficiently equipped in terms of facilities or personnel to manage possible severe allergic reactions.

2.3. Statistical analysis

Statistical analyses were performed by MedCalc software version 15.2.1 (Ostend, Belgium). Data were summarized as numbers (n) and frequencies (%) if they were categorical and as mean and standard deviation (SD) if quantitative. Comparison of two proportions was performed by Chi-square test with Yates' correction for continuity and comparison of two means by *t*-test. A *p*-value < 0.05 was considered statistically significant.

Relatedness of IgE recognition profiles was tested by applying unsupervised Eisen's hierarchical cluster methods {Eisen, 1998 40,305 /id} to the data set, encompassing allergenic molecules across all samples and using as agglomeration rule the average linkage clustering as implemented in the Genesis software {Sturn, 2002 29,824 /id}. Each square in the heat map represents the presence (red) or the absence (black) of IgE recognition of any given tested allergens for every single subject. The red colour intensity of every single square in the heat map is directly associated with the measured IgE concentration. Interpretation of the heat map generated by the software can be done either visually, where clustering IgE positive allergens tend to give more homogeneous red areas, or by taking into consideration the higher or lower level of dendrograms on the allergen side of the graph.

2.4. Ethics

All the tests were performed during routine care, and the samples were anonymized, since no personal data except for sex and age, was available. The Institutional Board of IDI-IRCCS confirmed that ethical approval was not required in this case (n. 493–1).

3 Results.

Out of the 56 subjects, 1 (1.8%) reported oral allergic syndrome (OAS), 28 (50%) experienced urticarial/angioedema, 18 (32.1%) anaphylaxis, 6 (10.7%) gastrointestinal symptoms alone and 3 (5.4%) respiratory symptoms alone. The culprit fishes are shown in Table 1. Cod (44.6%) and salmon (21.4%) were prevalent. Twenty-seven/56 (48.2%) showed symptoms on more than one occasion after ingestion of fish and 16/56 (28.6%) with more than one species. However, 29 patients did no longer eat fish following the first allergic episode. Twenty-nine subjects underwent SPT with commercial extracts (cod and/or salmon and/or tuna) and 22 (75.9%) scored positive. All seven patients who underwent PPT (4 cod, 1 cod + salmon, 1 tuna, 1 cod + trout + herring) scored positive. Altogether, 29/36 (80.6%) patients who underwent an in vivo test (either SPT or PPT) scored positive. Fifty-two out of the 56 subjects were tested for IgE to cod, salmon and tuna extracts: 48 (92.3%) resulted positive both for cod and salmon and 45 (86.5%) for tuna. The positivity for at least one of the three extracts was shown in 49 (94.2%) patients, which was significantly more frequent than that obtained with the SPT (*p* = 0.0397). Specific IgE levels to cod, salmon and tuna did not differ significantly in subjects with a history of anaphylaxis (8.22 ± 9.71 kUA/L, 11.56 ± 16.13 kUA/L and 6.29 ± 13.09 kUA/L, respectively) or with milder reactions (8.21 ± 10.22 kUA/L, 10.13 ± 14.79 kUA/L and 5.67 ± 11.8 kUA/L, respectively).

Table 1

Demographic and clinical characteristics of the studied patients.

	Number	Percentage (%)
Male/female	34/22	
Age (years)	mean 29.4 (range 2–80)	
<16 years	11	19.6
Culprit fish		
cod	25	44.6
salmon	12	21.4
tuna	4	7.1
trout	3	5.4
sole	2	3.6
gilthead bream	2	3.6
herring	2	3.6
swordfish	2	3.6
hake	1	1.8
pilchard	1	1.8
European bass	1	1.8
smoothhound	1	1.8
More than a culprit fish	16	28.6
Symptoms		
oral allergic syndrome (OAS)	1	1.8
urticaria/angioedema	28	50.0
anaphylaxis	18	32.1
gastrointestinal alone	6	10.7
respiratory alone	3	5.5

All the patients were tested for seven β -parvalbumins from different fish species, the α -parvalbumin from thornback ray, and the cod aldolase + enolase using the multiplexing assay. The positivity for the single β -parvalbumin Clu h 1, Cyp c 1, Gad m 1, Sal s 1, Sco s 1, Thu a 1 and Xyp g 1 was found on 42/56 (75.0%), 38/56 (67.8%), 35/56 (62.5%), 45/56 (80.4%), 45/56 (80.4%), 43/56 (76.8%) and 41/56 (73.2%) patients, respectively. Eight/56 (14.3%) patients scored positive for the α -parvalbumin (Raj c-parvalbumin), and 9/56 (16.1%) for the cod aldolase + enolase (Gad m 2 + 3). Six out of nine patients sensitized to Gad m 2 + 3 had cod as culprit fish. In two patients (3.6%) IgE to Gad m 2 + 3 was the only reactivity observed. The offending fish was salmon in one case and cod in the other. Fifty-two/56 (92.8%) patients scored positive to at least one β -parvalbumin, and 54/56 (96.4%) for at least one of the tested allergens (Table 2). The overlap among all the allergens is shown in Fig. 1.

The prevalence of Gad m 1 reactivity was significantly higher ($p = 0.01$) in cod reactors (84%) than in patients with a clinical history of reactivity to other fish species (48.4%). In salmon-allergic subjects, IgE to Sal s 1 were detected in 91.7% of cases vs 77.3% in subjects allergic to other fish species; the difference was not significant, likely for the low number of salmon reactors.

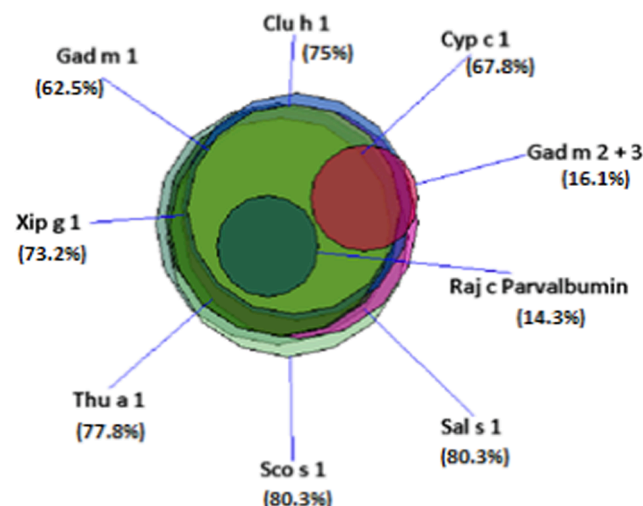
Interestingly, 3/4 tuna reactors, 2/2 herring reactors, and 2/2 swordfish reactors scored positive for Thu a 1, Clu a 1 and Xyp g 1, respectively. One of the patients who experienced a reaction with swordfish showed IgE positivity only for Xyp g 1.

No significant difference was detected between the number of positive allergens in patients who experienced anaphylaxis (mean: 6

Table 2

IgE recognition of molecular allergens of 56 fish allergic subjects.

Allergens	Positive number (%)
Gad m 1 + Gyp c 1	40/56 (71.4)
Gad m 1 + Cyp c 1 + Clu h 1	45/56 (80.3)
Gad m 1 + Cyp c 1 + Clu h 1 + Sal s 1	48/56 (85.7)
Gad m 1 + Gyp c 1 + Clu h 1 + Sal s 1 + Sco s 1	48/56 (85.7)
Gad m 1 + Cyp c 1 + Clu h 1 + Sal s 1 + Sco s 1 + Thu a 1	50/56 (89.3)
Gad m 1 + Cyp c 1 + Clu h 1 + Sal s 1 + Sco s 1 + Thu a 1 + Xyp g 1	52/56 (92.8)
All β -parvalbumins + Gad m 2 + 3	54/56 (96.4)
All β -parvalbumins + Ray c parvalbumin	54/56 (96.4)
All β -parvalbumins + Gad m 2-3 + Ray c parvalbumin	54/56 (96.4)

**Fig. 1.** Distribution of fish sensitization profiles in the study population.

allergens) vs those who experienced milder symptoms (mean: 5.1 allergens) ($p = 0.164$).

The Application of Cluster Analysis (Fig. 2) revealed a total independent IgE recognition profile of the β -parvalbumins tested when compared to the α -parvalbumin (Raj c-parvalbumin) and the mixture of cod aldolase + enolase antigens spotted on the chip. On the other hand, within the β -parvalbumins studied, Clupea harengus (Atlantic herring), Scomber sombrus (Atlantic mackerel), and Thunnus albacares (Yellowfin tuna) showed an almost identical IgE recognition profile, and all three clustered with Cyprinus carpio (Common carp).

4. Discussion

The diagnosis of fish allergy is more complex than other food allergies and, in some cases, it may represent an important challenge for the allergists due to the high variety of consumed fish species, the number of allergens that fishes contain, the methods of preparation of the diagnostic extracts that may alter the allergens, the low standardization of the extracts, and the limited panel of allergens available for diagnosis.

Some interesting aspects emerged from our real-life experience with fish allergy. First, the extracts for *in vivo* diagnosis showed an overall sensitivity of 75.8%, which is very similar to that observed in the study of Carvalho et al. [14], which found a sensitivity of 77% and 65%, using cod and salmon extracts, respectively. Instead, PPTs with the culprit fish showed an absolute sensitivity (100%) but were carried out only in 7 patients. Differences between SPT and PPT may reveal not only a potential lack of allergens in some commercial extracts but also that extracts from different species of fish do not react similarly.

Another aspect is that the *in vitro* diagnosis with cod, salmon and tuna extracts showed a higher overall sensitivity (94.2%) than the one observed with their *In vivo* counterparts. This could be explained on one hand by the higher sensitivity of the *in vitro* tests in the detection of low quantities of specific IgE and on the other one by differences in the process of preparation and purification of the extracts, which can lead to differences in the content of allergens, in particular of the β -parvalbumin. It is demonstrated that the concentration of parvalbumin varies from one part of fish to another: dark muscle contains less β -parvalbumin than white muscle and dorsal and rostral white muscles contain more β -parvalbumin than ventral and caudal white muscles [15]. For this reason, the use of different portions of fish meat for extract preparation may greatly influence the sensitivity of the tests. Moreover, the extraction techniques may lead to events such as fragmentation of linear epitopes or destruction of the conformational epitopes with a decrease in sensitivity.

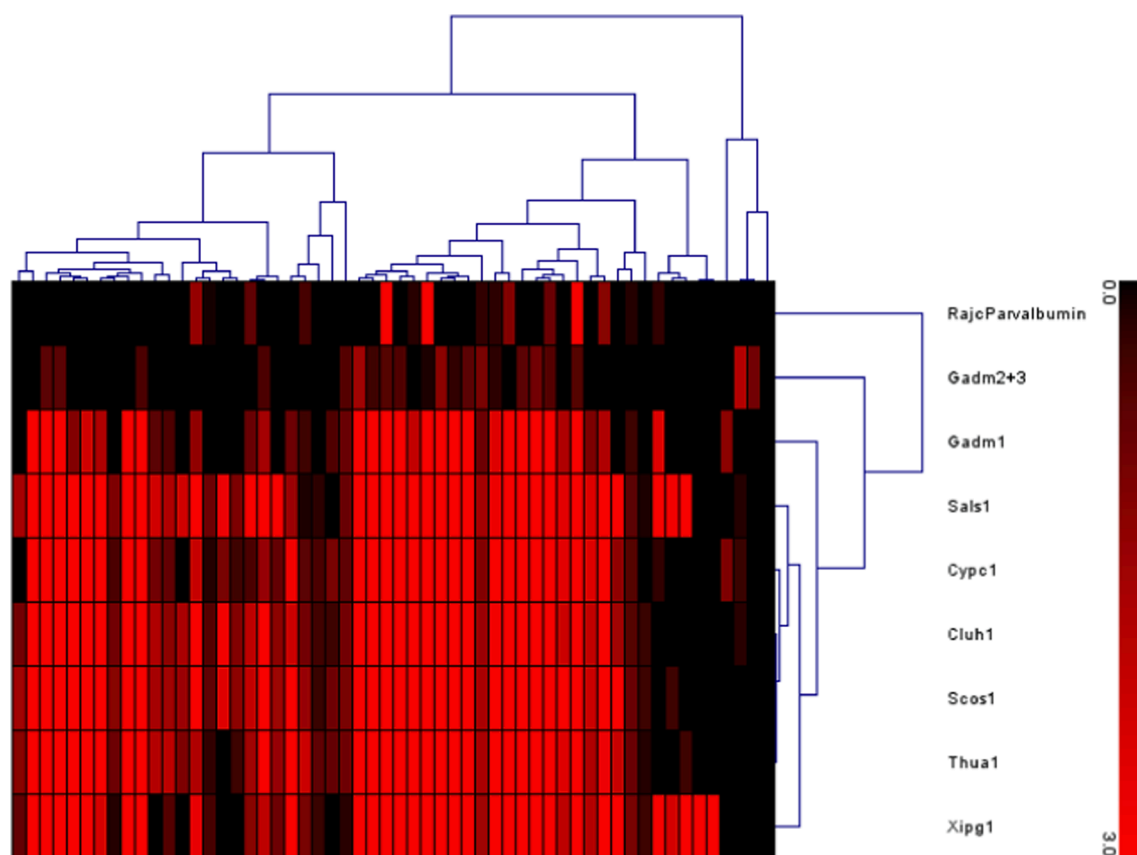


Fig. 2. Unsupervised bidirectional hierarchical clustering analysis generated different patterns of IgE reactivity in our population (x-axis) because of IgE recognition of Parvalbumins belonging to different biological sources (y-axis). Each square in the heat map represents the presence (red) or the absence (black) of IgE recognition of any given tested allergens for every single subject. The red color intensity of every single square in the heat map is directly associated with the measured IgE concentration (kU/L).

The third important observation regards the use of an expanded comprehensive panel of allergens for the molecular diagnosis of fish allergy. We confirm that β -parvalbumin represents the major fish allergen, but in our cohort, the prevalence of IgE reactivity ranged from 62.5% for Gad m 1 to 80.3% for Sals 1 and Sco s 1. These data show how IgE binding to various β -parvalbumin could be different, even if family members share sequence identities ranging from 63% to 88% [3]. Moreover, we demonstrated that in cod-reactors the prevalence of IgE reactivity to Gad m 1 was significantly higher (84%) than in patients reactive to other fish species (48.4%). Similar data were found in patients with a history of salmon-induced reactions where the IgE prevalence to Sals 1 reached 91.7%. As a whole, these data show that there is not a complete cross-reactivity among β -parvalbumins from different fish species; as a consequence a “one size fits all” approach, using only one or maximum of two β -parvalbumins available on singleplex method is not suitable for an accurate diagnosis of fish allergy. On the other hand, the use of a multiplexed assay gives the possibility to simultaneously detect IgE reactivity to various β -parvalbumins of the most representative fish species consumed in Italy, increasing the diagnostic sensitivity to 92.8%. For these reasons a CRD employing a wide range of β -parvalbumins selected from fish species covering the extent of a phylogenetic tree and representing the most consumed species in various geographic regions seems very important [16]. Moreover, the absence of specific IgE reactivity to β -parvalbumins of some species of fish could be a predictor of their tolerance, although to date oral food challenge remains the required method to rule out clinical reactivity. Further studies are needed to establish the usefulness of multiplex IgE testing for this purpose. In our study, only eight patients (14.3%) scored positive for α -parvalbumin and all were also reactive to β -parvalbumin.

These findings confirm on the one side the limited role of IgE to α -parvalbumin in the diagnosis of allergy to bony fish and on the other the existence of limited cross-reactivity between α - and β -parvalbumin due to their low amino acid sequence identity (<50%) [8]. In contrast, two out of the nine patients positive for Gad m 2 + 3 showed no other IgE reactivity and therefore the detection of IgE to Gad m 2 + 3 may increase the diagnostic sensitivity, demonstrating as their implementation in CRD can be useful to lower the rate of false-negative test results.

In conclusion, the multiplex test dosing an extended panel of specific IgE to β -parvalbumins from various species of fish, in addition to aldolase and enolase, shows high diagnostic sensitivity in fish allergy and it may represent a useful tool to overcome some potential problems related to the preparation and standardization of the fish extracts and the increasing lack of available extracts for SPT due to regulatory problems. Moreover, it might be used as an additional assay to investigate the cross-reactivity patterns between different molecular components. If different sensitization profiles may be a predictor of clinical reactivity or tolerance to different species of fish remains to be evaluated.

CRedit authorship contribution statement

Danilo Villalta: Conceptualization, Methodology, Writing – original draft. **Enrico Scala:** Conceptualization, Software, Resources, Writing – review & editing. **Gaia Deleonardi:** Investigation, Resources. **Maira Busa:** Investigation, Resources. **Federica Rivolta:** Investigation, Resources. **Elisa Boni:** Investigation, Resources. **Giulia Gabrielli:** Investigation, Resources. **Laura Farioli:** Investigation, Resources. **Camilla Cappelletti:** Investigation, Resources. **Francesca Buzzulini:** Investigation, Resources, Data

curation. **Mariaelisabetta Conte:** Investigation, Resources. **Valerio Pravettoni:** Conceptualization, Supervision, Writing – review & editing. **Riccardo Asero:** Conceptualization, Supervision, Writing – review & editing.

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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