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Multiparametric or multiplex systems in allergy diagnostics

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SUMMARY

Multiparametric or "multiplex" systems allow many and different biological molecules to be tested simultaneously "in vitro": there are basically two types, one is based on an $ISAC_{E112i}$ microarray technology (ISAC) and the other one on an $ALEX^2$ macroarray technology (ALEX). As for any immunodiagnostic assay, the two systems, ISAC and ALEX, are based on specific "designs" concerning the quality of the antigen, substrate, conjugated antibody and isotopic reference system used to calibrate the results. The use of multiplex systems in allergy diagnostics may be appropriate, unnecessary, or even harmful. An unnecessary and harmful use of multiplex systems can determine both risks for the patient such as the prescription of "absurd" extended exclusion diets, a self-diagnosis without a specialist visit, and social-economic risks with unjustified increase of costs by labelling patients as allergic when they actually are not. We have analyzed both these systems, highlighting their virtues, strengths and pitfalls, considering the currently available data in literature and the specific characteristics of each multiplex system. In order to avoid incorrect diagnosis and damage to patients, the choice between the two diagnostic tests has to be tailored to the patient and a prerogative of the specialist with specific knowledge in the field of molecular allergology and able to interpret the results.

KEYWORDS: ALEX, CCD, IgE, ISAC, multiplex, singleplex

INTRODUCTION

Multiparametric or multiplex systems allow numerous and different biological molecules to be tested "in vitro" simultaneously. In allergy diagnostics there are basically two types: the first is based on an $ISAC_{E112i}$ (ISAC) microarray technology and the second on $ALEX^2$ (ALEX) macroarray technology. Both systems are able to generate a report with a broad profile of the patient's IgE sensitization for over 100 allergenic molecules, allowing first and foremost to distinguish genuine sensitizations from cross-reactivities.

Like any immunodiagnostic assay, the two systems, ISAC and ALEX, are based on specific "designs" with regards to the quality of the antigen, substrate, conjugated antibody, and isotopic reference system used to calibrate the results. In allergy diagnostics these aspects are particularly critical and it has, in fact, been frequently observed that different serological dosing systems generate non-overlapping and non-interchangeable results [1-3].

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CHARACTERISTICS

The ISAC system was the first multiparametric system available for allergy diagnostics and has been continuously updated since 2001. Today it allows the dosage of 112 selected monomolecular IgE from 48 different allergenic sources from the animal and plant world. Thanks to microarray technology, it requires microscopic quantities of biological sample and reagents, and carries out all assays in triplicate on a microchip to always guarantee a reliable result. The fluorescence reading system includes a confocal laser scanner.

The more recent ALEX system, together with the dosage of 178 monomolecular IgE, also combines the dosage of 117 polyclonal IgE towards complete allergenic extracts. Another 5 monomolecular IgE can also be measured by the system for research purposes only and are not validated for clinical diagnostics. The reading system uses an optical density colorimetric reader.

Table I shows the salient characteristics of the two diagnostic systems [4,5]. In particular, those technical and analytical peculiarities that have an important role in the "performance" of immunodiagnostic systems and can favor their use in certain situations were compared. The width of the measurement range of the results, the sensitivity of the reading system, the ability to eliminate interferences that could lower the specificity of the test, and the number and quality of the molecules immobilized on an adequate support, are all aspects which play a fundamental role in the performance of the analytical system and the wishes of the clinical allergist.

CORRELATION STUDIES

When comparing the two ISAC and ALEX systems, it must first be considered that the technical and methodological characteristics are different. In fact, the two solid support phases for the antigen have substantially different chemical-physical peculiarities, and the production of allergenic extracts and molecular components has a different origin. The ALEX methodology requires significant pre-dilution of serum and the unit of measurement and the marking system of human anti-IgE for the clarification of the reading signal is different, and as a consequence the system for reading the results is different.

ALLERGENIC EXTRACTS

In analytical studies, regarding the dosage of IgE towards allergenic extracts, the ALEX system has been compared to "singleplex" ImmunoCAP technology which should be considered the "gold standard" of in vitro diagnostic tests [2,6]. In comparing the results relating to inhaled allergenic extracts, in an Italian study on a selected population of 105 allergic patients, ALEX showed a semi-quantitative correlation κ (Cohen's kappa coefficient) of 0.64. In the case of food allergenic extracts, ALEX showed a significantly lower semi-quantitative correlation: $\kappa = 0.47$[7].

MOLECULAR COMPONENTS

In other publications, where the two systems relating to the most important molecular "markers" of sensitization were directly compared, non-linear correlations between ALEX and ISAC were shown. In a Czech study on a sample of 198 patients, due to the reduced reading "range", the ALEX system showed problems of underestimation for values higher than 20 kU/L, in particular with regard to the following sensitization "markers" primary: Bet v 1 (birch), Der p 1 and Der p 2 (house dust mite), Phl p 1, Phl p 5 (mouse tail), and Fel d 1 (cat)[1].

Other comparisons on the molecular components have shown a good quantitative correlation between the two systems, although in

TABLE I. Comparison between the main characteristics of $ISAC_{122i}$ and $ALEX^2$.

	$ISAC_{122i}$	$ALEX^2$
Current version	2019	2019
Type of array	Microscopic	Macroscopic
Sample volume (microliters)	30	100
Measurement range	0.3-100	0.30-50
Reading signal	Fluorometric	Colorimetric
Solid phase support	Microchip in glass slide	Nitrocellulose chip
Elimination of CCD interference	100% (out of 108 on 112 components)	85-95%
Predilution necessary	No	Yes 1:5
Number of molecules	112	178
Number of complete extracts	0	117
Total IgE	No	Yes

an Italian study on a population of 43 allergic patients, in the case of important "markers" of sensitization, such as Ara h 8 and Ara h 9 (peanut), Cor a 1 (hazelnut), Gly m 4 (soya), Jug r 2 (walnut), Phl p 2 and Phl p 5 (rat tail), the coefficient correlation "r", although significant, was less than 0.8 [8].

In a European multicenter study, the Spearman's correlation coefficient (rs) was evaluated for the six molecular allergens considered causal in the context of the so-called "pollen food syndrome": Mal d 1 (apple), Api g 1 (celery), Cor a 1 (hazelnut), Ara h 8 (peanuts), Gly m 4 (Soya), Hev b 8 (Latex). On a population of 53 consecutive patients with suspected allergic reactions, the rs coefficients ranged from 0.943 for Mal d 1 to 0.757 for Api g 1 [9].

A recent Italian multicenter comparative study on molecular components compared the ALEX system to the ISAC system on a selected population of 140 allergic patients. Considering ISAC as the "gold standard" for "multiplex IgE arrays", a qualitative correlation κ=0.795 was shown. The qualitative comparison analysis (negative/positive results) shows a concordance of 71% if negative concordant results are excluded. The agreement expressed as a correlation coefficient of the quantitative data is less than 0.9 (0.824). The data set was defined as "not comparable" [10]. Also in this study, the correlation coefficient showed a moderate relationship due to the lower reading range of the results of the ALEX system which flattens all responses above 40 kU/L. The graphs reported in the study show that the ALEX system almost never reads results above a value of 35 kU/L and this generates a moderate relationship in terms of intraclass correlation coefficient (ICC). Finally, the Bland-Altman graphical analysis showed the absence of correlation that was probably due to the different measurement intervals of the two methods [10].

More recently, in a Dutch study carried out on a population of 49 samples found to be polysensitized according to previous analyses, ISAC reported an ICC = 0.58 [11]. In the same study, the quantitative comparison confirmed the "bias" between the two dosing systems with a "plateau" of the ALEX system when results approach 50 kU/L. This last aspect is also reported in the comparative analyses divided into groups of allergens such as animal epithelia, grass pollen, tree pollen, and the various molecular components studied, including PR-10 (Bert v 1 homologues), Ole and 9 (olive), and LTP (Lipid Transfer Protein) [11].

RESOLUTION STRATEGIES FOR POSSIBLE INTERFERENCES FROM IGE ANTI-CROSS-REACTIVE CARBOHYDRATE DETERMINANTS (CCD)

There are different effective approaches to decrease or eliminate the risk of false positivity from CCD and therefore increase diagnostic accuracy [2]. Currently, three different strategies are followed to avoid possible interferences in "in vitro" tests due to the possible presence of IgE CCD in blood:

1. production of recombinant molecular allergens without carbohydrate components;
2. use of blood sample diluters that inhibit CCDs during the assay process;
3. use of MUXF3 (an allergenic component equipped exclusively with carbohydrate epitopes present in numerous plant glycoproteins) as a positive control for CCDs.

The ISAC system follows the first strategy coupled with the third, thus guaranteeing the absence of possible interference at the source and at the same time measuring the concentration of specific IgE CCDs.

The ALEX system, following the second strategy coupled with the third, involves a preliminary methodological phase of the sample with a serum diluter that is 85% effective in inhibiting antibodies for CCDs [5]. Therefore, a large part of the possible interference caused by IgE specific for the cross-reactive carbohydrate determinants, possibly present in the sample, is cancelled [8]. However, in a recent publication the blocking of anti-CCD antibodies in the ALEX system was found in 60% of the cases studied (33 samples reactive to MUXF3 in the ISAC system) [10]. It is useful to point out that the problem of CCDs, except for 4 native glycoprotein molecular components, is absent in ISAC technology.

CONSIDERATIONS

The application of "multiplex" technology to "in vitro" allergy diagnostics represents a step forward in defining the patient's sensitization profile with a molecular perspective in both respiratory and food allergies. Consequently, this has led to solving difficult clinical cases such as complex polysensitizations and idiopathic anaphylaxis with greater precision. However, the ease with which this type of test can be requested even in non-specialist contexts represents a potential risk of abuse, as it should never be considered a first level investigation. On the contrary, prescription of the "multiplex" test should be the prerogative of those who have sufficiently high knowledge of molecular allergology and are able to appropriately interpret the different and sometimes complex sensitization profiles.

Numerous publications from different countries, although characterized by their own "setting" for the diagnosis of respiratory and food allergic diseases, have provided a qualitative and quantitative comparison framework between the two "multiplex" technologies best known today: ISAC and ALEX. While considering dichotomized results, through a qualitative analysis, a significant correlation emerges between the two technologies, and the comparisons between quantitative measures show a partial agreement between the two systems for both the primary sensitization "markers" and the panallergens [10]. A limitation of "multiplex" methodologies is that they are semi-quantitative and less sensitive than "singleplex" methodologies [7,8]. Their reduced ability to precisely quantify each IgE clone certainly represents one of the reasons why the results of the different systems are not interchangeable [12-14]. The lower sensitivity of

the "multiplex" methodologies is highlighted even more heavily on the "performance" of IgE measurement for extracts where the choice of the allergenic substrate is particularly critical.
The two "multiplex" systems taken into consideration herein are based on specific "designs" for the quality of the antigen, the chemical-physical characteristics of the substrate, the immuno-chemical characteristics of the conjugated antibody, and the isotype reference system used for the calibration of the results. Since these aspects are crucial in allergy diagnostics, it has not been infrequently observed that different serological dosages on "multiplex" systems generate non-overlapping and non-interchangeable results.
The appropriate use, and not abuse, of these new technologies also requires a good level of knowledge of the methodology used. For this purpose, especially for pediatric patients, priority should be given to the reduced volume of serum, the wide reading range of the results, the calculation of results from "multiplets", and correlation with ImmunoCAP technology considered the "gold standard" of tests "in vitro" allergy diagnostics. Finally, and certainly not of less importance, it is essential for the allergist to know the allergens present in the different platforms and what impact the new allergenic molecules that are proposed can have on diagnosis and clinical decisions. In pediatric allergology, especially in the diagnosis of food allergy such as for example in the eczematous symptoms of children, the "multiplex" test can provide information on sensitization levels of unknown clinical relevance, which are not useful either for appropriate modifications of the diet or for the suggestion of extra-oral provocation tests [15].
Aiming at a real enhancement of diagnostic appropriateness and an improvement in the efficiency of the National Health Service, the development of new diagnostic technologies and the proposal of new monomolecular IgE dosages on "multiplex" systems should always be supported by scientifically adequate documentation on the "performance" and clinical significance of the molecular components.

CONCLUSIONS

The use of "multiplex" systems in allergy diagnostics is:

1. appropriate when prescribed and used in patients with complex poly-sensitizations and idiopathic anaphylaxis, when it is necessary to investigate latentizing co-factors or hidden allergens;
2. useless when prescribed before specialist medical examination, when anamnesis and clinical history have already outlined the clinical case, when targeted molecular diagnostics ("singleplex") have comprehensively defined the clinical picture;
3. harmful when the person prescribing it lacks adequate knowledge of "Molecular Allergology" to correctly interpret the results and develop the appropriate diagnostic and therapeutic approach, when the patient receives a complex report without a clinical interpretation (e.g. the sensitization is different from allergy, from illness), when performed as a first level examination before specialist medical examination.

Unnecessary and harmful use of "multiplex" systems can lead to risks for the patient such as, for example, the prescription of "absurd" extended exclusion diets, self-diagnosis of allergy by the patient himself, based only on laboratory tests, without having first carried out specialist evaluation, and carries social-economic risks with unjustified increase in costs by labeling a patient as allergic who in reality is not.
Based on the analysis of the currently available data in the literature and the specific characteristics of each of the available "multiplex" systems, we have analyzed both systems highlighting their merits and strengths. Very importantly, in order not to make incorrect diagnoses and cause damage to the patient, is that the choice of which of these two diagnostic tools to use is tailored for the patient and is a prerogative of the specialist with specific knowledge in the field of molecular allergology and able to interpret the results.By personalizing the choice for the individual patient, the opportunity to carry out in-depth molecular diagnostic tests and, based on specific needs, the specialist can choose whether to carry out multiparametric analysis or tests for single allergenic molecules. With a view to a strengthening the diagnostic appropriateness and an improvement in terms of effectiveness of the National Health Service, it is to be hoped that the development of new diagnostic technologies and the proposal of new monomolecular IgE dosages on "multiplex" systems are always supported by scientifically adequate documentation on the "performance" and clinical relevance of the molecular components.

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Conflicts of interest statement

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Ethical consideration

The article is unpublished, not simultaneously submitted to another journal and complies with current legislation on research ethics.

Author's contribution

RB: conceptualization. RB, SA, SB, DC, FC, GD, AG, SG, CM, LP: data curation and writing. RB: writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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