



Multiplexed analytical approaches to beta-lactam allergy in vitro testing standardization



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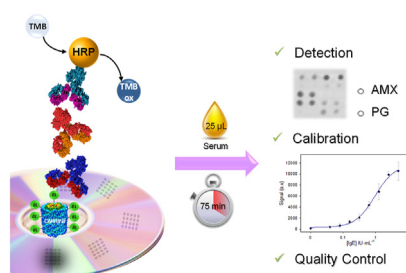
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HIGHLIGHTS

- The impact of the homologous calibration is demonstrated by testing specific IgE to β -lactam antibiotics.
- Internal homologous calibration provides inter-method comparison with 0.99 agreement for penicillin G and amoxicillin.
- A chimeric IgE is used as internal standard to improve the analytical performance of the β -lactam in vitro testing.
- A sensitivity of 144 ng/L, 2-fold better reproducibility and 93–106% of accuracy is reached.
- A reliable medical care at the point-of-need to expand delabeling initiatives in a cost-effective way is achieved

GRAPHICAL ABSTRACT



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ABSTRACT

The suspicion of beta-lactam allergy directly contributes to the prescription of antibiotics that diverge from the guidelines, increasing antimicrobial resistance, one of the biggest threats to global health. In vitro quantification of specific IgE is very useful for monitoring allergy, as it confirms or rules out immediate beta-lactam drug allergy and helps find safe alternative antibiotic stewardship. However, reliable in vitro quantification of specific IgE to beta-lactam antibiotics by immunoassay is challenging because of the difficulty of having selective immunoreagents, mainly beta-lactam antigens, and its low concentration levels in serum. Thus, reliable and sensitive in vitro tests for multiplex detection of allergy to different beta-lactam antibiotics is currently essential for clinical diagnosis. Nevertheless, the lack of standardization of quantitative in vitro methods makes the comparison and interpretation of the results difficult. Here, as proof of concept, we report an improved multiplex microimmunoassay for beta-lactam allergy in vitro testing standardization. The results revealed that homologous calibration allows reliable

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quantification of specific IgE in human serum at very low concentrations (144 ng L^{-1}). Moreover, the reproducibility of the results increases 2-fold using an internal standard, achieving accurate quantitative information: 93% and 106% recovery for penicillin and amoxicillin, respectively. We simultaneously evaluated the reliability of the improved multiplexed in vitro method in a cohort of 40 human serum samples and achieved excellent agreement (0.99) with a currently used in vitro test.

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1. Introduction

Beta-lactam (BL) antibiotics are the most frequent cause of immunoglobulin E (IgE)-mediated drug hypersensitivity. Hence, current in vitro diagnostic tests are intended to determine serum levels of specific IgE (sIgE). Previous studies have revealed a significant correlation between sIgE concentration and the severity of allergy symptoms [1]. Consequently, from a clinical perspective, in vitro quantification of drug-sIgE is essential, which however is currently not possible in a reliable way due to the lack of specific calibrators. Moreover, the standardization of β -lactam allergy in vitro testing is still an unmet need.

There are semi-quantitative analysis methods, such as the RAST inhibition and ImmunoCAP-ISAC assays, each with their own units [2–4], making the comparison between them difficult. The conventional calibration approach for a quantitative assay without a specific standard is based on the heterologous model, a mean that uses the Third WHO International Standard as the calibrator for total IgE quantification [5]. Interpolation from the heterologous calibration curve allows antigen-sIgE quantification [6]. Some of the reference systems for IgE quantification using heterologous calibration models exhibit good correlation with certain allergens, but frequently there are disparities in the results (a correlation between 0.53 and 0.73) because of the allergens used and the lack of specific calibration standards [7,8]. In fact, most of the in vitro tests available show very low sensitivity (<30%) and give results that differ from test to test what makes the results difficult to interpret.

Several studies have showed that in BL allergy, the protein-hapten antigen is key for sIgE detection, providing results that may differ on the carrier molecule [9], the molar ratio [10], and the hapten employed [11,12]. Consequently, heterologous calibration generates discordant values between in vitro diagnostic tests since there are no reference antigens nor specific standards. So that, there is a diagnostic need to harmonize testing protocols for the diagnosis of β -lactam allergy.

To establish a standardized in vitro test system for drug allergy, very few studies have used the homologous calibration approach to quantify sIgE concentrations with a representative positive serum sample as calibrator or standard [13,14]. Although this calibration approach has been shown to have higher correlation compared to other methods, it requires large amounts of human serum with sIgE for each BL-antigen to establish a reference material. Another drawback of in vitro BL allergy tests is the lack of multiplexing to evaluate the response in patients with allergies to different antibiotics. Although multiplex assays have many advantages, they usually have lower linear range, cross reactivity with other antibodies, such as IgG or IgM [15], and higher coefficient of variation [16], as observed with ImmunoCAP-ISAC testing [2]. In addition, with current in vitro tests, periodic calibration curves must routinely be constructed to preserve the accuracy and precision of the assay. Though commercially available tests exist, protocols are not standardized between laboratories in terms of antigens and analytical procedures; they are expensive and require equipment

that is not available in all test centers and they present some disadvantages or technical limitations such as its low sensitivity.

Overall, there is a real need for accurate and validated assays to precisely control serum sIgE levels to BL antibiotics. Therefore, current limitations must be solved in order to develop sensitive in vitro tests for reliable determinations of sIgE to different drug allergens. Here, we present improved analytical approaches for penicillin-G (PG) and amoxicillin (AMX)-sIgE quantification, standardizing sIgE determination and providing a solution to the main current calibration issues. We developed heterologous and homologous external calibration strategies and compared their accuracy and precision. Moreover, internal calibration methods were also studied to improve the analytical features of the multiplex in vitro test and reduce the quantities of the required standard. For that, a BL antigen microarray format was developed that included positive controls as internal calibrators and an internal standard for quantitative calibration. As proof of concept, precise and sensitive simultaneous determination of drug sIgE is presented. The reliability of the analytical approaches was evaluated in a cohort of 40 human serum samples, and the results compared with the in vitro reference test.

2. Materials and methods

2.1. Reagents, buffers and instruments

Human AB serum, human serum albumin (HSA), and Histone Type III-S (H1) were purchased from Sigma-Aldrich (Madrid, Spain); penicillin G sodium salt, 98% (PG), AMX, 96%, and aztreonam (AZT) from Acros Organics (Geel, Belgium); recombinant human IgE (rIgE), and goat anti-mouse IgG preabsorbed (GAM-HRP) from Abcam (Cambridge, United Kingdom); monoclonal anti-human IgE (α IgE) from Ingenasa (Madrid, Spain) and omalizumab, a recombinant humanized monoclonal antibody that selectively binds to human immunoglobulin E, from Novartis International AG (Basel, Switzerland). The WHO reference IgE standard 11/234 was provided by the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom). Artificial human serum (cIgE) was obtained through the fusion of the Fc domain of IgE and Fc ϵ RI (CD64) with polyclonal specific IgG to AZT as described elsewhere [17] and employed as the internal standard. Antigenic conjugates, H1-AMX, and H1-PG were used as antigens⁹. In-house reference material was obtained from a representative pool of positive samples with known IgE concentrations to AMX ($5.09 \text{ IU} \cdot \text{mL}^{-1}$) and PG ($4.37 \text{ IU} \cdot \text{mL}^{-1}$), determined by the ImmunoCAP reference method.

The following buffers were used: printing buffer (50 mM sodium carbonate, pH 9.6), conjugation buffer (0.5 M sodium carbonate, pH 11.0), purification buffer (PBS 1X (8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl), and washing buffer (PBS-T (8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, 0.05% (v/v) Tween-20, pH 7.4)). Buffers were filtered through $0.45 \mu\text{m}$ pore nitrocellulose membranes before use. The antibody dilution buffer and ep(HS)TMB-mA colorimetric substrate were purchased from SDT-reagents (Baesweiler, Germany).

2.2. Human serum samples

Forty serum samples from adult subjects were used in this study, 19 from allergic patients and 21 controls. Samples were characterized based on patient's clinical history, result of prick tests to different BL antibiotics, and reference in vitro test (ImmunoCAP). Serum samples from allergic and control patients were extracted and characterized following standardized protocols by Hospital Universitari i Politènic La Fe (Valencia, Spain) and then aliquoted into vials and frozen at -60°C until use. An IgE-free serum control sample from a patient suffering from IgE deficiency disorder was used as diluent to prepare the calibrators for heterologous calibration. All participants were enrolled after giving written informed consent according to protocols approved by the ethics review board at Hospital Universitari i Politènic La Fe (registry no. COBIOPHAD). Procedures were followed in accordance with the Helsinki Declaration of 1975, as revised in 2008. Patients were diagnosed as per the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, in vitro tests, or drug provocation test, whenever necessary.

2.3. Protocol for the assay

The assay consisted of a direct multiplexed test using a DVD (CD Rohling-up GmbH, Saarbrücken, Germany) as the analytical support to determine sIgE (Fig. 1A). First, 50 nL of the BL antigens or antibodies diluted in printing buffer or PBS, respectively, were dispensed into the DVD surface with a non-contact printing device (AD 1500 BioDot, Inc., Irvine, CA), generating spots of $550 \pm 38 \mu\text{m}$ in diameter (0.24 mm^2) with a track pitch (center to center distance) of 1.0 mm, achieving an array density of 1.27 spot/mm^2 . Human serum albumin (40 mg L^{-1}) was used as the negative

control, and recombinant human IgE at different concentrations ranging from 0.625 to 10 mg L^{-1} as positive controls for internal calibration. The layout of the array consisted of five rows of two reagents each with two replicates. The printing scheme of the DVD is shown in Fig. 1B. Twenty microarrays were spotted on a disc and the DVD was incubated at 37°C overnight. Next, $25 \mu\text{L}$ of sample (raw human serum or the WHO standard for specific and total IgE determination, respectively) were dispensed over the array and incubated for 30 min. Sera samples were spiked with $4.5 \text{ IU}\cdot\text{mL}^{-1}$ of cIgE used as the internal standard. After incubation, the solution was washed with PBST and de-ionized water. Next, $25 \mu\text{L}$ of 1.0 mg L^{-1} of the monoclonal anti-human IgE were dispensed onto the array and incubated for 15 min. After washing, 5.0 mg L^{-1} of goat anti-mouse IgG preabsorbed were added and incubated for 15 min. Finally, the immuno-interaction was developed using $25 \mu\text{L}$ of the substrate (ep(HS)TMB-mA) for 15 min. The reaction was stopped by washing the surface with deionized water.

2.4. Calibration strategies

In this study, we assessed five calibration strategies: 1) heterologous and 2) homologous as external calibrations models, 3) heterologous and 4) homologous internal standardization using positive controls, and 5) internal standard calibration. The different calibration approaches are depicted in Fig. 2.

The heterologous calibration (1) was performed using a sandwich immunoassay with a matching pair of monoclonal anti-IgE antibodies and the WHO IgE standard serially diluted in human IgE-free serum as the calibrator. The signal obtained for each calibrator was fitted to a four-parameter logistic curve. The homologous calibration approach (2) consisted in a direct assay with the antigens immobilized on the disc surface in microarray format.

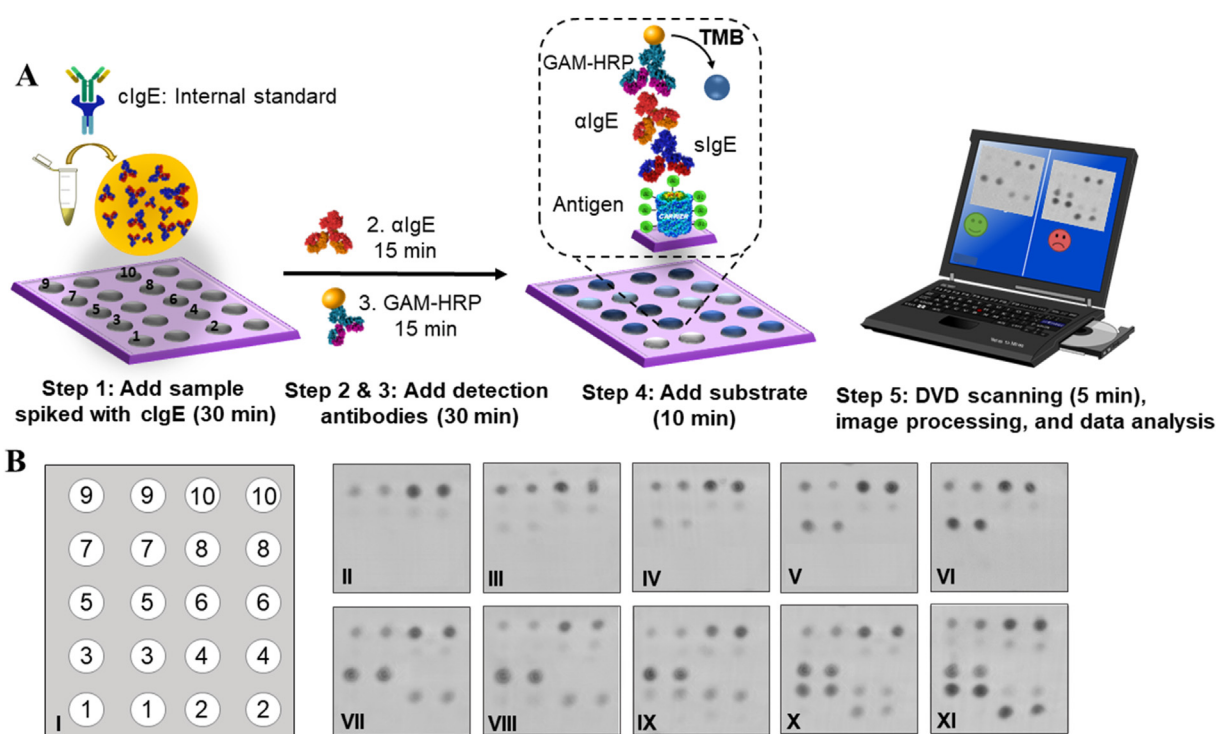


Fig. 1. (A) Schematic representation of the multiplexed assay. (B) Images of the BL array. Panel I shows the layout of the array (spots 1 to 10): 1: Negative control, 2–4: penicillin, amoxicillin, and aztreonam antigens, respectively 5: Omalizumab, 6–10: IgE (0.625 , 1.25 , 2.5 , 5 , and 10 mg L^{-1} , respectively). Panels II–VI show the results of the assays for the heterologous calibration methods (1 and 3) using the WHO standard as the sample (0 , 0.1 , 0.4 , 1.6 , and $6.4 \text{ IU}\cdot\text{mL}^{-1}$, respectively). Panels VII–XI depict the results of the homologous calibration methods (2, 4, and 5) using an in-house reference material obtained from a representative pool of positive samples (0 , 0.06 , 0.3 , 1.1 , and $4.4 \text{ IU}\cdot\text{mL}^{-1}$ for penicillin G and 0.07 , 0.3 , 1.3 , and $5.1 \text{ IU}\cdot\text{mL}^{-1}$ for amoxicillin).

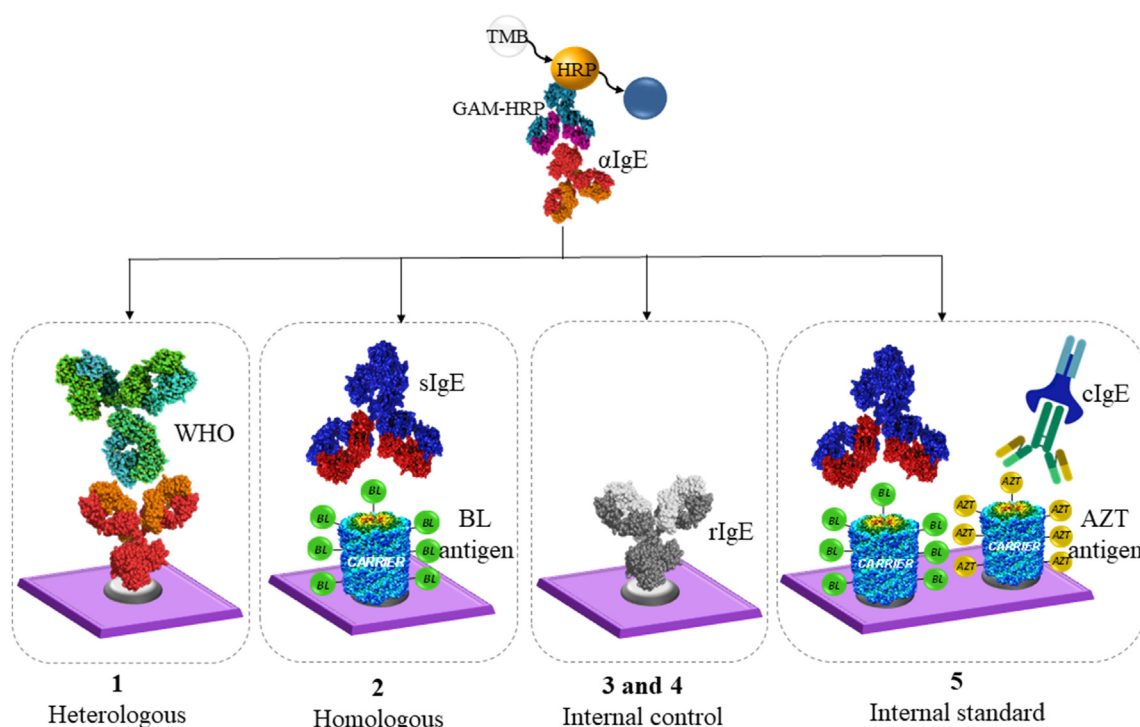


Fig. 2. Schematic representation of the five calibration approaches.

Calibrators were obtained from a serial dilution of an in-house reference material diluted in a pool of negative control human serum sample. As for the calibration approach (1), the signal obtained for each calibrator was fitted to a four-parameter logistic curve. The calibration methods (3) and (4), internal heterologous and homologous calibrations, respectively, were based on the use of positive controls printed on the microarray. The signals given by the positive controls were linked to those of the calibrators of the heterologous and homologous approaches to build the corresponding calibration curves. For the calibration method (5), an internal standard was used. For that, the calibrators obtained from a serial dilution of a representative pool of positive samples were spiked with $4.25 \text{ IU} \cdot \text{mL}^{-1}$ of cIgE. The following equation was used to calculate the normalized signals:

$$\text{Normalized signal} = \text{Signal of BL-antigen} / \text{Signal of AZT-antigen}(1)$$

2.5. Data analysis

Optical results were measured using a modified commercial disc drive as described elsewhere [18]. Briefly, a DVD player is connected to a computer through a USB 2.0 connector and managed with custom software. The DVD reader, equipped with a laser, emits at 650 nm. The laser interrogates the surface of the DVD and an optoelectrical sensor picks up the reflected beam. During DVD scanning, the laser beam strikes the bluish immunoreaction product, which modifies the reflection properties of the DVD surface. Consequently, the intensity of the laser beam is absorbed and the optoelectrical sensor detects an attenuated reflection signal. The variation in the reflected signal intensity is proportional to the IgE concentration, that is, the higher the IgE concentration, the greater the attenuation of the laser beam intensity detected by the sensor. The data collected by the sensor were digitized and converted into images (one image per each printed array). The image of the array

was processed, and signals quantified using a software customized by our laboratory (BioDisk). The analytical signal was calculated by subtracting the local background from the median signal value of the spot.

2.6. Analytical features

Analytical sensitivities were expressed as limit of detection (LOD) and limit of quantification (LOQ). These parameters were calculated measuring 10 times a pool of 16 negative serum samples and interpolating the mean of the signal plus 3 or 10 times the standard deviation of the negative controls for LOD and LOQ, respectively, into each calibration curve, providing a concentration value for IgE.

Intermediate precision (inter-disk) was established considering sample measurement deviations in duplicate on six different days and expressed in terms of relative standard deviation (RSD). Mean IgE concentrations were compared against those obtained by the ImmunoCAP reference system, accepting this value as the real concentration of sIgE to assess the accuracy of the method. Accuracy was expressed in terms of recovery (%) and calculated with the following equation:

$$\text{Recovery (\%)} = (\text{sIgE determined by DVD system} / \text{sIgE determined by ImmunoCAP}) \times 100 \quad (2)$$

Furthermore, precision and accuracy of each quantification system were also assessed by examining the relationship between the values obtained by the respective quantification and those of the reference method (Pearson correlation).

3. Results and discussion

3.1. External calibration approaches

BL-sIgE concentrations in quantitative reference methods are

currently calculated by interpolating the signal in a total IgE calibration curve. Although heterologous calibration is the most accepted scheme to measure sIgE, the trend of an IgE antibody to bind to a specific epitope has not been well addressed. To demonstrate this, we built heterologous and homologous calibrations for PG and AMX. The results were fitted into four-parameter logistic curves (Fig. 3).

The half-maximal effective concentration (EC_{50}) and slope for each curve were compared using the ANOVA test. No significant differences ($p = 0.1590$) between the two calibration approaches were detected for PG, contrary to what was observed for AMX-sIgE quantification, for which significant differences were observed ($p = 0.0036$), with overestimated values for IgE concentrations when following the accepted heterologous scheme. The differences between both curves corroborate that the trend of an IgE antibody to bind to a specific epitope is key for reliable quantitative information. Regarding sensitivity, the calibration approaches showed a LOD and LOQ of $0.10 \text{ IU} \cdot \text{mL}^{-1}$ and $0.41 \text{ IU} \cdot \text{mL}^{-1}$ for PG, respectively. However, homologous calibration for AMX improved the detection and quantification limits to 0.06 and $0.22 \text{ IU} \cdot \text{mL}^{-1}$, respectively. These results confirm that the apparent affinity constant is antigen-dependent, and heterologous calibration may affect the accuracy of the method for AMX-sIgE quantification. Assay reproducibility in terms of RSD was between 20% and 35% for both calibrations.

3.2. Internal calibration approaches

According to the results obtained with the external calibration approaches, standardization of IgE concentration is better with the homologous strategy, ensuring compatibility with other allergy quantification methods. However, the main drawback is that, as it occurs with other microarray-based methods, regular calibration curves need to be plotted. For this purpose, large volumes of representative serum samples containing high amounts of sIgE are needed, a request, which is not always feasible. We overcame this limitation using internal calibration approaches through internal positive controls within the microarray as the calibration strategy [19]. Recombinant IgE was chosen as the most significant positive control within the microarray. Different IgE concentrations (0.625 – 10 mg L^{-1}) were immobilized on a disc (see Fig. 1B, spots 6 to 10). The signals for each concentration were interpolated into the corresponding external calibration curves, making internal heterologous and homologous curves available. Inter-disk internal calibration RSDs were calculated by testing six disks, 20 arrays per disk, and two replicates per array (240 spots); variations between 11% and 23% were found for the different positive control IgE

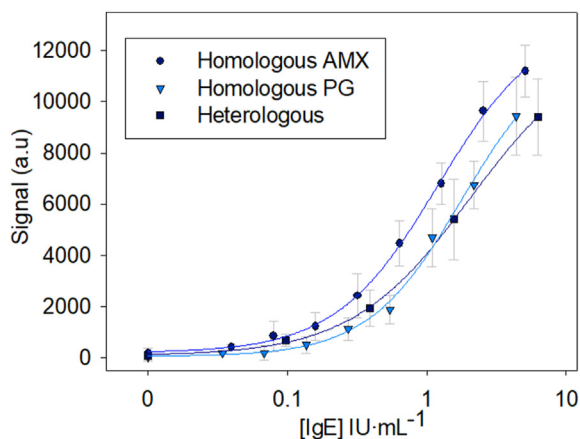


Fig. 3. Calibration curves for the heterologous (1) and homologous (2) approaches ($n = 30$).

concentrations (Fig. S1). These results demonstrate that this calibration strategy is robust and allows performing easy internal calibrations for each individual sample, correcting systematic errors of the immunoassay.

Another calibration approach was based on the use of internal standards, which may be advantageous for reliable multianalyte quantitation. This method is widely used in chromatography and mass spectroscopy to accurately quantify compounds in samples. With the aim to normalize signals, the internal standard is added to all samples and calibration materials in a constant known quantity. This enables to compensate or reduce some errors that may occur in the measurement procedure or that may affect the DVD system such as errors in the pipetting of samples, compensation for fluctuations in the analytical signal that may occur in the sensor, and compensation for a possible matrix effect, among others. An AZT-specific artificial serum (clgE) with the same physical and chemical properties of sIgE was used as the internal standard; however, the artificial serum recognizes a unique AZT epitope and does not interfere with the determination of sIgE to PG and AMX.

To confirm that clgE is a good internal standard and get the most out of the analytical feature of this approach, the following four parameters were evaluated: optimal spike concentration, selectivity of the compound, compatibility with the sample, and stability in human serum samples. First, different concentrations of clgE were added to a representative serum sample with known sIgE concentrations to AMX and PG and then it was evaluated following the immunoassay protocol described in materials and methods. Results are shown in Fig. S2. Based on the value of the analytical signal (corresponding to the EC_{50} of the calibration curve) and reproducibility (15% and 8% inter-disk and intra-disk, respectively), $4.25 \text{ IU} \cdot \text{mL}^{-1}$ was selected as the optimal concentration. The internal standard only recognizes the AZT antigen (Spot 4) (Fig. 1B, panel VII), while for the PG and AMX antigens (Spots 2 and 3, respectively) negligible signals were detected. The stability of the internal standard in human serum was also assessed. For this study, human serum, spiked with $4.5 \text{ IU} \cdot \text{mL}^{-1}$ of clgE, was incubated for different times before performing the immunoassay. No statistically significant differences between signals were found within 4 h of incubation (Fig. S3), although after 8 h, a decrease was observed. This implies that clgE was stable and compatible with the human serum sample under the assay conditions (total assay time is less than 2 h). Moreover, negligible signals for the AZT antigen for serum samples from patients and controls were obtained, indicating absence of AZT-sIgE in serum. These results confirm that clgE can be used as an internal standard for PG- and AMX-sIgE quantification.

Standardized homologous calibration curves using the internal standard clgE for PG- and AMX-sIgE determinations show (Fig. 4) a sensitivity that is similar to that obtained in external homologous calibration for both antibiotics (LOD for PG and AMX 0.12 and $0.06 \text{ IU} \cdot \text{mL}^{-1}$, respectively). Regarding reproducibility, the RSD varied from 10% to 15%, 2-fold better than with external calibration approaches. We increased the reproducibility of the results and thus, the reliability of the analytical information.

The robustness of this approach was also evaluated by spiking representative serum samples containing 2.4 , 1.2 , 0.6 , and $0.3 \text{ IU} \cdot \text{mL}^{-1}$ of PG-sIgE and 1.76 , 0.88 , 0.44 , and $0.22 \text{ IU} \cdot \text{mL}^{-1}$ of AMX-sIgE with the internal standard ($4.25 \text{ IU} \cdot \text{mL}^{-1}$), analyzed in three replicates. Spiked samples were also diluted (50–75%) to evaluate the possible experimental errors on sIgE quantification associated with sample handling. As shown in Table 1, correct IgE quantification was possible using external calibration, although experimental errors were not corrected. By contrast, the introduction of an internal standard in the sample provided accurate determination of PG and AMX-sIgE concentrations, regardless of the degree of

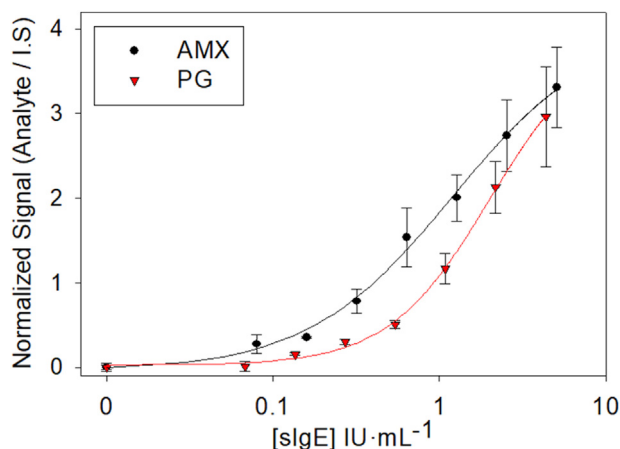


Fig. 4. Calibration curves for amoxicillin- and penicillin G-specific IgE quantification using the internal standard approach.

experimental error (recovery of 71–127%).

The tendency of IgG to bind to the immobilized antigens is well-known and reported for the commercial ImmunoCAP ISAC assay [3]. Thus, interference with slgE binding -present in lower concentrations-occurs, with the consequent decrease of the sensitivity of the assay. Moreover, IgG concentration may differ between patients, affecting the accuracy of IgE quantification. Standardizing the response among patients to obtain reliable values is key. For this purpose, a sample with high concentration of slgE for PG was diluted in three matrices: raw human serum, partially depleted human serum (free from IgG), and PBST. The results are depicted in Fig. 5A. As expected, improvement in IgE recognition by the antigens was achieved by removing IgG from the serum. However, the depleted serum still contained other interferences affecting recognition. The effect of the matrix was thus normalized by spiking the samples with 4.25 IU·mL⁻¹ of the internal standard (Fig. 5b); no significant differences were found between the normalized signals for each concentration ($p = 0.100$), establishing that this approach generates a single response regardless the composition of the sample. Furthermore, these findings indicate the versatility of the method by allowing to evaluate samples with different matrix composition and generating accurate results without the need to recalibrate or optimize the system.

3.3. A side-by-side comparison of the calibration methods

To evaluate the analytical features of the calibration methods, a cohort of 40 human serum samples were evaluated, 19 allergic

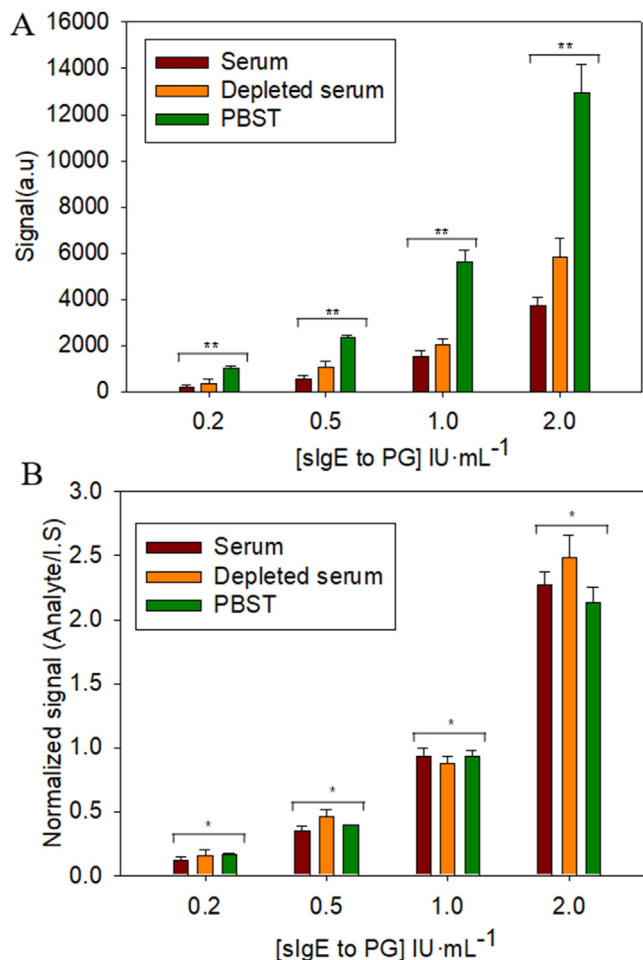


Fig. 5. (A) Effect of sample matrix on penicillin G-slgE recognition signal. (B) Effect of sample matrix on penicillin G-slgE recognition signal after the normalization with the internal standard *No statistically significant differences **statistically significant differences.

patients and 21 controls. Calibration modes are highly influenced by precision; we carried out a comparison of their mean RSDs. For PG-IgE determination, both external calibration approaches showed high RSD, 38% and 35%, probably due to the requirement of periodic recalibrations (Table 2 and Fig. S4A). Nevertheless, the internal calibration approach improved the precision, reaching 30, 22, and 14% for the methods (3), (4) and (5), respectively. Similar trends were observed for AMX-slgE determination (Table 2 and Fig. S4B).

Table 1

Comparative results between external and internal standard slgE quantification.

Sample lost (%)	Penicillin G (IU·mL ⁻¹)				Amoxicillin (IU·mL ⁻¹)			
	2.41	1.21	0.60	0.30	1.76	0.88	0.44	0.22
0	2.90 ± 0.93 ^a 2.78 ± 0.40 ^b 115 ^c	1.18 ± 0.30 1.24 ± 0.19 103	0.76 ± 0.10 0.74 ± 0.07 123	0.34 ± 0.11 0.29 ± 0.03 97	1.77 ± 0.27 ^a 1.74 ± 0.17 ^b 99 ^c	1.08 ± 0.25 0.83 ± 0.11 95	0.59 ± 0.13 0.51 ± 0.12 115	0.19 ± 0.06 0.25 ± 0.05 111
50	1.36 ± 0.44 ^a 2.71 ± 0.29 ^b 112 ^c	0.48 ± 0.12 1.33 ± 0.20 110	0.29 ± 0.04 0.59 ± 0.05 97	0.08 ± 0.03 0.36 ± 0.04 120	0.99 ± 0.15 ^a 1.73 ± 0.17 ^b 99 ^c	0.39 ± 0.09 0.78 ± 0.11 89	0.25 ± 0.07 0.47 ± 0.11 108	< LOD 0.24 ± 0.04 110
75	0.39 ± 0.13 ^a 2.15 ± 0.31 ^b 89 ^c	0.22 ± 0.06 0.86 ± 0.13 71	0.13 ± 0.02 0.69 ± 0.06 114	< LOD 0.38 ± 0.04 127	0.44 ± 0.07 ^a 1.82 ± 0.28 ^b 103 ^c	0.11 ± 0.03 0.79 ± 0.11 90	< LOD 0.48 ± 0.11 110	< LOD 0.27 ± 0.05 123

^a External homologous calibration (2).

^b Internal standard calibration (5).

^c Recovery (%) for internal standard calibration. LOD: limit of detection.

Experimental concentrations calculated by interpolation of the signals on the different calibration curves were compared with the IgE measurements obtained using the current reference system (ImmunoCAP) (Pearson correlation). As observed in Table S1 and Fig. S5 and S6, external calibrations (1 and 2), showed correlations of 0.958 and 0.961 for PG, and 0.857 and 0.928 for AMX, respectively (Pearson correlation). On the other hand, better correlations with the reference methods were obtained when internal calibration approaches (3), (4), and (5) were employed, achieving linear correlations of 0.991, 0.983, and 0.985, for PG, and 0.989, 0.993, and 0.989 for AMX. The accuracy of the systems was also examined based on the slope of lineal regression and the percentage of recovery. The best accuracies are seen for calibration methods (4) and (5), being the slope close to 1 and the recovery around 100%. For the rest of the calibration methods, although a slope of around 1.1 and a recovery percentage between 73 and 105% for PG-sIgE determination was achieved, slope values for AMX-sIgE determination were 1.23, 0.79, and 2.10 and with recoveries of 168, 82, and 217% for methods (1), (2), and (3), respectively (Table 2 and Table S1). Based on the results, we established that for PG, all calibration methods show valid results and a high degree of accuracy. On the other hand, heterologous approaches 1 and 3 overestimate AMX-sIgE concentration (168% and 217%). Therefore, the performance of the homologous calibration methods is better than that of the heterologous for a multiplexed assay. According to precision and accuracy results, calibration methods 4 and 5 have the best performance. Moreover, the calibration using the internal standard shows better robustness, good precision, and excellent accuracy.

Similar clinical performance results were obtained for the different calibrations models (Table S2-S5), achieving a clinical sensitivity of 42 and 95% for PG and AMX, respectively (Fig. 6). Compared to the reference method, 1.5 and 1.7-fold improvements in clinical sensitivity were obtained for PG and AMX, respectively, due to the improved calibration mode. Negligible signals were observed in all the controls analyzed, thereby establishing a clinical specificity of the system of 100%.

4. Conclusions

In comparison to recent results, this manuscript reports unprecedented new analytical approaches and advances to standardize beta-lactam allergy in vitro testing. To the best of our knowledge, this is the first time that an internal standard and homologous calibration approach are used, contributing to the improvement of analytical performance for the precise determination of IgE specific to β -lactam antibiotics. The internal standard allows compensating or reducing the analytical errors that can occur in the measurement procedure. Ultimately, this enables the development of a reliable in vitro test in microarray format for the multiplex diagnosis of drug allergy. As shown in Table 3, the

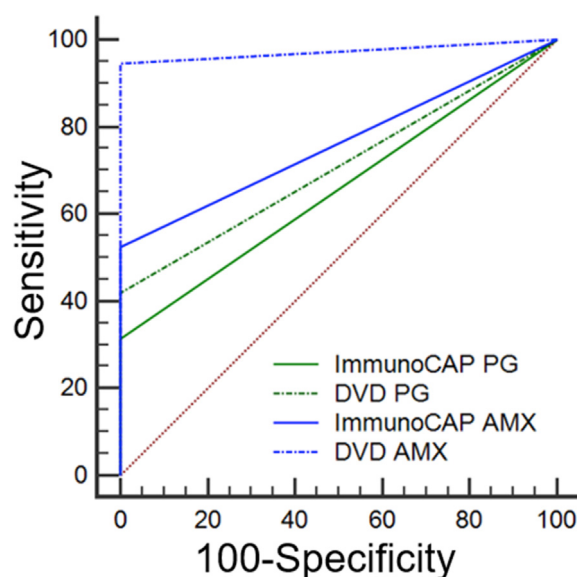


Fig. 6. Receiver operating characteristic (ROC) analysis for DVD technology and ImmunoCAP for penicillin G and amoxicillin ($n = 40$ results).

developed test based on the homologous approach achieved better correlation with the reference system than those based on heterologous calibration. Furthermore, our test improves the diagnostic sensitivity without reducing the specificity and allows the detection of allergy to several β -lactams simultaneously.

The homologous calibration with a pool of representative positive samples from allergic patients is a suitable approach to standardize sIgE quantification particularly to AMX, as demonstrated in terms of analytical performance and increased sensitivity of the assay.

Overall, the DVD multiplexed immunoassay described in this study simultaneously determines sIgE to BL antibiotics with high clinical and analytical sensitivity. It provides the possibility of coupling standardization approaches, improving the analytical performance of the assay to reliably determine sIgE present at very low levels without the need of periodic external calibrations. In summary, we have established a suitable multiplexed immunoanalytical approach to precisely determine sIgE below $0.1 \text{ IU} \cdot \text{mL}^{-1}$ with only $25 \mu\text{L}$ of serum in a total assay time of 1 h. Moreover, thanks to multiplexing, the immunoassay allows screening patients with allergies to multiple antibiotics, which would provide valuable information when deciding which drug to prescribe. Besides, this approach allows the evaluation of a wide range of patients with very low specific IgE concentrations and different recognition patterns, and in a cost-effective manner showing excellent

Table 2

Comparisons of analytical parameters for the five calibration methods.

Calibration method	BL	LOD ($\text{IU} \cdot \text{mL}^{-1}$)	Sensitivity ($\text{IU} \cdot \text{mL}^{-1}$)	Precision (%)	Accuracy (%)	Robustness ^a
1	PG	0.12	2.0	38	76	+
	AMX	0.12	2.0	39	168	
2	PG	0.14	1.8	35	73	++
	AMX	0.06	1.0	35	82	
3	PG	0.12	2.0	30	105	++
	AMX	0.12	2.0	30	217	
4	PG	0.14	1.8	22	85	+++
	AMX	0.06	1.0	32	98	
5	PG	0.12	1.5	14	93	++++
	AMX	0.06	1.0	20	106	

^a Classification made based on the precision and accuracy results. LOD, limit of detection.

Table 3Comparison of performances of in vitro diagnostic testing for β -lactam allergy.

Cut-off (IU/mL)	Sensitivity (%)	Specificity (%)	Multiplex	Calibration	Detection	Correlation ^a	Reference
2.5%	43–75	68–83	No	External	Radioactive	ND	[20]
0.35	10–50	80–98	No	Heterologous	Fluorescence	–	[21,22],
0.35	9	100	No	Heterologous	Chemiluminescence	<0.8	[22,23],
0.25	ND	ND	No	Homologous	SPR	0.99	[13]
0.05	65	100	No	Heterologous	Chemiluminescence	0.87	[14]
0.06	90	100	Yes	Homologous	Colorimetric	0.99	This work

^a ImmunoCAP was used as the reference method for correlation studies. ND: not determined.

agreement (0.99) with the currently gold standard in vitro test.

Our findings reveal that the unique multiplexed compact disc-based microimmunoassay quantifies sIgE of the most common BL antibiotics, allowing reliable medical care at the point-of-need for patients with a history of drug allergy to BLs. The beta-lactam allergy multiplexed in vitro testing standardization might improve the reliability of clinical diagnosis, providing clinical evidence required to support the decision-making of the allergist, inform allergen-avoidance regimes, ameliorate the design of personalized therapeutic strategies, and advance the β -lactam delabeling initiatives in clinical practice.

CRediT authorship contribution statement

María José Juárez: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Ethel Ibáñez-Echevarría:** Conceptualization, Investigation, Resources, Data curation. **Dolores Hernández-Fernández de Rojas:** Resources, Data curation. **Ángel Maquieira:** Conceptualization, Project administration, Writing – original draft, Funding acquisition. **Sergi Morais:** Conceptualization, Methodology, Supervision, Project administration, Writing – original draft, All authors have given approval of the final version of the manuscript.

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.aca.2021.338656>.

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