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A multiparametric highly sensitive chemiluminescence immunoassay for quantification of β-lactam-specific Immunoglobulin E

Pedro Quintero-Campos[1], María José Juárez[1], Sergi Morais[*1,2,3], Ángel Maquieira[1,2,3]

[1]Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València-Universitat de València, Camino de Vera s/n, (46022) Valencia, Spain.

[2]Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, (46022) Valencia, Spain.

[3]Unidad Mixta UPV-La Fe, Nanomedicine and Sensors, IIS La Fe, Av. Fernando Abril Martorell, (46026), Valencia, Spain

Author e-mail: smorais@upv.es;

ABSTRACT: β-lactams (BLCs) are the most widely used antibiotics, and consequently the most common cause of drug allergy in the world. The diagnosis of drug allergy is complex and represents a serious challenge that includes a wide variety of methods. *In vitro* tests are based on immunological determination of allergen-specific IgE, but the tests in the market lack the required sensitivity and specificity. Also, the large sample volume, long incubation times and single-plex configuration have brought their use into question to complement the clinical information. Here, we report a chemiluminescence immunoassay (CLIA) for multiparametric quantification of specific IgE to penicillin G, penicillin V, amoxicillin and piperacillin, using histone H1 as carrier. The developed CLIA allowed the determination of BLCs-specific IgE below 0.1 IU/mL, allowing identifying allergic patients with better sensitivity, using only 25 µL of sample (serum). The immunoassay was successfully applied in a cohort of 140 human serum samples, showing good sensitivity (64.6 %) as well as specificity (100 %), what significantly improve the predictive character of existing BLCs-allergy *in vitro* tests.

INTRODUCTION

β-lactams antibiotics (BLCs) are the most widely used drug to fight bacterial infections worldwide due to their efficacy, safety and price, representing 65% of the world antibiotic market[1]. Among the BLCs, penicillin, amoxicillin and amoxicillin-clavulanate are the utmost prescribed[2]. However, it is estimated that these antibiotics are the most common cause of drug allergy in the world, with prevalence ranging from 5% to 10% in the general population[3] and have an approximate frequency of 81% of all allergic drug reactions in children[4]. The overall consumption of antibiotics ranged from 4.4 to 64.4 Defined Daily Doses (DDD) per 1000 inhabitants per day[2], what might make allergy episodes to occur.

One of the four types of allergy is associated to IgE-mediated type I hypersensitivity reactions and its diagnosis is complex and represents a serious challenge that includes a wide variety of methods[5]. *In vivo* methods (skin test or drug provocation test) are invasive and risky of a new allergic reaction, even causing anaphylaxis in the most severe causes[6]. On the other hand, *in vitro* methods are based on the detection of specific IgE and are useful in the identification of the causative culprit drug without any risk[6]. In fact, it is the only method able to determine directly the levels of drug-specific IgE (sensitization). A positive specific IgE response accompanied with a history of allergic symptoms make the diagnosis of drug allergy clinically relevant[7].

In recent years, a variety of *in vitro* tests have been developed that can determine the level of specific IgE in blood or serum for food and environmental allergens. The vast majority of these methods are multiplex and they are based on the simultaneous determination of specific IgE against different allergens. Some of them are AdvanSure AlloScreen Max (LG Chem), Polycheck Allergy (Biocheck), ALEX Allergy Explorer (Macro Array Diagnostics) and IVD Capsule Aeroallergens (Abionic SA). These methods require specific and expensive equipment, their test times can reach more than three hours and they use large volumes of sample. Despite differences in the methodology, these methods have very similar characteristics, with detection limits in the 0.35 IU/mL level and high clinical sensitivity[8–10]. However, *in vitro* tests for drug allergy diagnosis to β-lactams are scarcely developed.

In clinical practice, a low variety of *in vitro* serological tests have been developed to detect specific IgE to β-lactam antibiotics in serum, including RAST (Radio Allegro Sorbent Test), fluorescence enzyme assays (as ImmunoCAP) and ELISA (Enzyme linked immunosorbent assay)[11].

RAST method is outdated due to the inconvenience of using radioactive isotopes, inefficiency and high cost[12]. This assay has been replaced by fluoroenzyme assays as ImmunoCAP (Thermo Fischer Scientific), which is perceived as the reference method for the *in vitro* detection of specific IgEs. However, ImmunoCAP does not cover all allergens and the cut-off value considered positive is 0.35 kUA/L. Despite their differences, IMMULITE (Siemens) is another method to detect specific IgE. Both methods have many weaknesses such as test time, sample volume, the semiquantitative character, the cost per assay-allergen[13] and are far from representing an effective and reliable alternative to *in vivo* tests, showing a poor sensitivity of 25%[3,14].

This makes that even though approximately 15% of the world population are labelled as allergic to BLCs[15], most diagnoses of β-lactams allergy are related to events that are not allergic in nature, and therefore, are associated with negative clinical and

administrative outcomes, including use of less desirable alternative antibiotics, longer hospitalizations, increasing antibiotic-resistant infections, and greater medical costs[16]. BLCs allergy de-labelling has become a global operationalizing focused to avoid unnecessary treatment and inferior results with alternative agents as well as adverse public health outcomes such as antibiotic resistance[17].

Consequently, there is a clinical-commercial demand for new diagnostic methods that meet the requirements of sensitivity, specificity, speed, simplicity, for their implementation in all types of clinical laboratory settings of different levels of health care.

ELISA has been widely used as a sensitive and selective method in analytical assays, so it can represent a valid alternative to FEIAs, being relatively simple and inexpensive for the assessment of serum sIgE for common allergens[7]. However, this method often requires large volumes of sample and consumes long incubation time in the whole process. Nevertheless, assay optimization processes could lead to very sensitive and selective ELISAs using less sample volume and taking less test time. At last, one alternative to improve immunoassay sensitivity is to incorporate chemiluminescence into the standard ELISA protocols (CLIA). This technique has the potential to improve sensitivity by at least 2-3 orders of magnitude compared to conventional colorimetric detection[18]. In this type of assays, luminol is used as substrate for the HRP-labelled antibody[19].

In addition, this technology allows estimating the sensitization profile of each patient, enabling individualized and precision therapy, in line with the emerging strategy aimed at focusing on the individual characteristics of each patient for prevention, diagnosis and treatment of the illness.

To the best of our knowledge, we here report the first multiparametric CLIA-based method for the *in vitro* determination of specific IgE for amoxicillin, penicillin G, penicillin V and piperacillin IgE-mediated drug allergic reactions for commonly prescribed and consumed β-lactam antibiotics.

MATERIALS AND METHODS

Reagents, buffers, consumables and instruments

Histone H1, penicillin G, penicillin V, amoxicillin and piperacillin were from Sigma-Aldrich (Madrid, Spain). Omalizumab was from Novartis International AG (Basel, Switzerland). WHO reference IgE standard 11/234 was from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom). Mouse monoclonal antibody anti-human IgE (α-IgE) was from Eurofins Ingenasa S.A. (Madrid, Spain). Goat anti-mouse IgG preabsorbed (GAM-HRP) was purchased from Abcam (Cambridge, United Kingdom). Enhanced chemiluminescent substrate solution was acquired from Thermo Fisher (Madrid, Spain). Coating buffer was 50 mM sodium carbonate/bicarbonate, pH 9.6, and washing buffer 10 mM sodium phosphate buffer, 150 mM NaCl, 0.05% Tween 20%, pH 7.4 (PBS-T).

High binding white polystyrene ELISA plates were from Costar Corporation (Cambridge, MA, USA), the ELISA plate washer from Nunc Maxisorp (Roskilde, Denmark) and the EnSpire Multimode Plate Reader from PerkinElmer (Waltham, MA, USA).

Preparation of coating antigens

β-lactam-lloyl antigens were prepared following the method described by Edwards with slight modifications[20]. Briefly, β-lactam antibiotics (penicillin G, penicillin V, piperacillin and amoxicillin at 50 µmol) reacted with the carrier protein (H1 at 0.25 µmol) in 1.5 mL of 0.1 M sodium carbonate, pH 11.0 overnight at room temperature. The antigens were purified by centrifugal filters (Amicon Ultra centrifugal filters) using PBS (10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2) as buffer exchange. The antigens were stored at -20 ºC till use.

Assay procedure for multiparametric chemiluminescence immunoassay

A scheme of the chemiluminescence immunoassay procedure is depicted in Figure 1. First, white flat-bottomed polystyrene ELISA plates were coated with antigen solutions (3.0 mg/L) in coating buffer (25 µL/well) for the direct determination of specific IgE to β-lactam antibiotics. Also, 25 µL of omalizumab solution (0.5 mg/L in coating buffer) was used to coat wells as the capture antibody for the determination of total IgE concentration in a sandwich format for calibration purposes. The plates were then sealed and incubated overnight at 4 °C. The following day, plates were washed four times with PBS-T and after that 25 µL/well of sera and WHO standards were added to each well, followed by incubation for 30 min at room temperature. Serum samples were analyzed in triplicate and sIgE-free serum (H4522, Sigma-Aldrich) was used as negative control. One calibration curve (WHO standards in triplicate) was made on each ELISA plate. Then, the wells were washed as before. Next, 25 µL of monoclonal antibody anti-human IgE solution (1/2000 dilution) was added to each well. After 15 min, the plate was washed as before and 25 µL of goat anti-mouse IgG preabsorbed solution (1/500) were added to each well and incubated again for 15 minutes. After washing the wells as before, the peroxidase activity was measured by adding 25 µL of enhanced chemiluminescent substrate solution previously diluted 1/10 in PBS. The luminescent signals were read at 450 nm, using the Multimode Plate Reader.

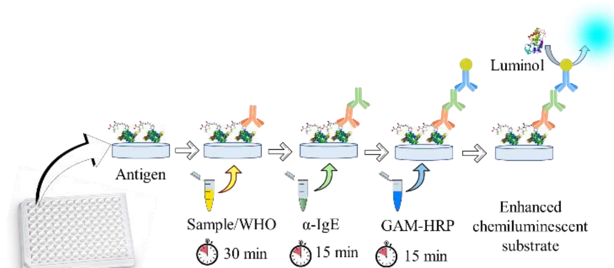

Figure 1. Scheme of the multiparametric chemiluminescence immunoassay in a 96-well microplate for the determination of specific and total immunoglobulin E (IgE) to β-lactam antibiotics in human serum.

Allergen-specific IgE levels expressed as IU/mL were determined, using the WHO standards, involving heterologous interpolation as a calibration method. The limit of detection (LOD) and quantification (LOQ) was calculated measuring the signal of the blank ten times and interpolating the mean of the signal plus 3 and 10 times the standard deviation to the calibration curve, respectively. Likewise, a representative serum sample with known specific IgE concentration, measured by the reference *in vitro* ImmunoCAP method, was used as calibrator to obtain a homologous calibration curve for analytical comparison purposes.

Patients

Serum samples from 140 adults were collected in red-top tubes (BD Diagnostics, Madrid, Spain), incubated at room temperature for 60 minutes to induce clotting. After centrifugation at 2000 rpm for 15 minutes, the serum was aliquoted into cryovials and stored at -80ºC till use. A cohort of 71 allergic patients (allergic to at least one of the four β-lactam antibiotics under study) and 69 non-allergic to BLCs individuals (controls) were included in the study. The clinical history of the patients included the result of the prick test to different ß-lactam antibiotics and the concentration of specific IgE measured by the ImmunoCAP test. All participants were enrolled after giving written informed consent according to protocols approved by the ethics review board at La Fe University Hospital (registry no. COBIOPHAD). The procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 2008. The patients were diagnosed following 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.

Statistical analysis

Coefficients of variation and parameters of assay sensitivity were determined by standard descriptive statistical methods using Microsoft Excel 365 (Microsoft Corporation). The standard data points were plotted and a four-parameter logistic (4PL) curve was fitted through the points, using SigmaPlot 12 (Systat Software Inc). ANOVA statistical analysis, multiple regression analysis and the correlation study were carried out using SPSS Statistics (IBM). Clinical sensitivity and specificity were calculated using MedCalc (MedCalc Software) using ImmunoCAP as reference method.

RESULTS AND DISCUSSION

Assay optimization

The time and reagent volume required to perform an ELISA test are the main reasons why this type of assays is not commonly used for allergy diagnosis. To overcome these two disadvantages, an assay optimization study was carried out. Histone H1-Amoxicillin conjugate (H1-AMX) was chosen as antigen and a well-characterized serum sample, with known concentration of specific IgE to amoxicillin (3.3 IU/mL, according to the ImmunoCAP results) was used. This study was carried out, using 3-fold serial dilutions (1/3-1/27) made up with control serum samples from non-allergic patients.

First, the blocking step was excluded. The assay was compared with and without a blocking step, and statistical analysis of the data through a multiple regression analysis using SPSS Statistics (IBM) showed that there were no statistically significant differences between both methodologies (p=0.265) (Figure S1). This might be probably due to the blocking effect of serum proteins.

Secondly, a decrease in incubation time and sample volume required to perform the test was carried out. The goal was to move from a 3 hour-assay, the time usually taken to perform a regular ELISA, to 65 min-assay; 30 min for serum sample incubation step, and 15 min for both the primary and secondary antibody incubation.

The volume of both the reagents and sample as well as the total assay time were evaluated by carrying out four assays: 100 µL of sample and each reagent, and a total assay time of 3 hours (standard protocol); and 50, 25 and 10 µL of sample and each reagent with a one-hour total assay time. Once the test was performed, as can be seen in Figure 2, a proportional reduction in the luminescent signal was observed when the test volumes and time decreased.

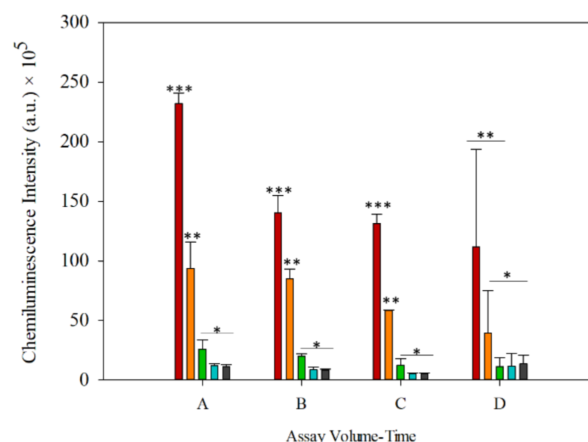

Figure 2. Results of the optimization assays (A-D) with Histone-AMX as antigen and a representative positive serum sample. A: Standard protocol (100 µL of volume used and total assay time 3 h). B, C and D: 50, 25 and 10 µL of volume used, respectively, and total assay time was approximately 1 h. The group of bars (A-D) correspond to specific IgE concentration to amoxicilin [IgE]. From the left to the right the concentration was 3.3; 1.1; 0.35; 0.12 and 0 IU/mL.

By analyzing each of the experiments separately, the data statistics show that when 10 µL of sample is used there is no statistically significant difference between the average light signal and the different IgE concentrations. When the volume used to perform the ELISA is 100, 50 and 25 µL the ANOVA statistical analysis establishes the existence of statistically significant differences in the light signal as the specific IgE concentration varies.

To compare the use of different volumes, a multiple regression analysis was performed. It established that the only variable that produced statistically significant changes in the light signal (dependent variable) was the specific IgE concentration ($p < 0.05$), and that both the volume (p=0.210) and the time of test performance (p = 0.810) did not significantly influence the final result.

So, taking into account all the results, the test was switched to use a volume of 25 µL and 1h total assay time. Compared to other immunoassays with the same number of steps, high sensitivity was achieved with reduced incubation time. In addition, the volume of 100 µL typically used in a standard ELISA protocol has been reduced from the 50 µL of serum required to perform IgE analysis with IMMULITE (Siemens)[21] or the 40 µL used in the most up-to-date ImmunoCAP (Phadia) equipment[22].

Evaluation of antigens performances

Antigens are the essential element to develop a sensitive and selective assay for the determination of specific IgE antibodies to antibiotics. This is because BLCs are low-molecular-weight molecules incapable of inducing an immune response on their own. Protein haptenization is the process that occurs through the nucleophilic opening of the β-lactam ring and results in protein-antibiotic antigens capable of triggering the immune response[6]. For this reason, the carrier molecule is key to induce

sensitization and consequently it is responsible to activate the immune system in order to produce specific IgE antibodies against a particular epitope. In this study, histone H1was used as carrier molecule to conjugate the β-lactam antibiotics. Histone H1 is a lysine rich protein that contains tens of primary amines available for coupling β-lactam antibiotics, enabling high-yield conjugations.

Linearity-of-dilution tests were carried out for validating and assessing the accuracy of CLIA, using the H1-AMX antigen. These tests were performed with a reference serum with known concentration of specific IgE for amoxicillin (7.0 IU/mL as measured by ImmunoCAP), using 3-fold serial dilutions (1/3-1/81). Dilutions were made with a pooled (n = 69) control serum sample as diluent. As is shown in Figure 3, the linearity was good over a wide range of dilution, revealing that the methodology provided flexibility to test human serum samples with different levels of specific IgE. The lowest specific IgE concentration to amoxicillin likely to be reliably distinguished from the diluent (negative control) was 0.03 IU/mL.

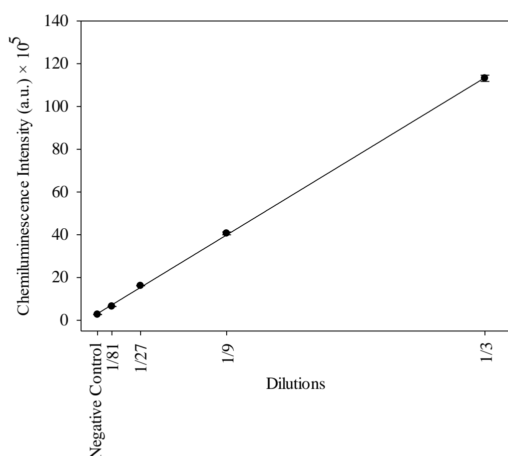

Figure 3. Dilution linearity study (r^2 = 1) of the assay using a serum sample as matrix to evaluate the accuracy of the CLIA.

The rest of the histone derived antigens (penicillin G, penicillin V and piperacillin) performed well, showing a LOD of 0.03 IU/mL for penicillin G, V, calculated from the results obtained with the linearity-of-dilution experiments (Figure S3).

Calibration method

A reliable quantitative method must report the results in units traceable to an international standard. Nowadays, it is not possible to use individual specific IgE for β-lactam antibiotics to perform homologous calibration method. Therefore, the calibration method mostly accepted is a heterologous interpolation of specific immunoglobulins E from a single total immunoglobulin reference curve. As it can be observed in Figure 4 (solid line), the signal (n=10) fit well to a four-parameter logistic curve, achieving a limit of detection of 0.05 IU/mL and dynamic response ranging from 0.5 IU/mL to 8.0 IU/mL, following a point-to-point calculation method approach. On assessment of the precision of the CLIA, CVs ranged from 1.6 to 8.6% and resulted in a linear regression equation with correlation coefficient (r) of 0.999. Likewise, a representative sample with known concentration of amoxicillin-specific IgE was used for a homologous calibration method, using the H1-AMX antigen. As it can be observed in Figure 4 (dash line), the signal (n=6) fit well to a four-parameter logistic curve, achieving a limit of detection of 0.03 IU/mL. In the homologous curve, CVs ranged from 0.5 to 2% and resulted in a linear regression equation with correlation coefficient (r) of 1.

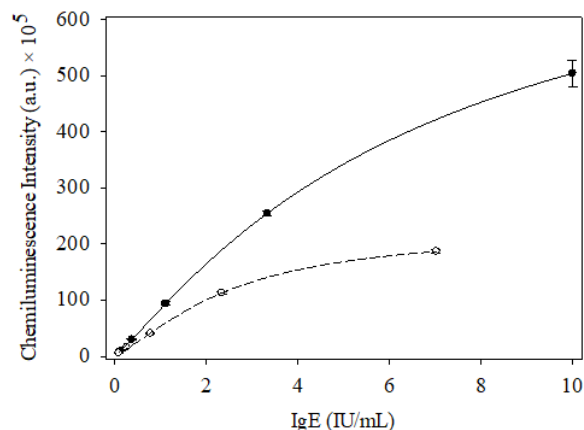

Figure 4. Heterologous (solid line) and homologous (dashed line) calibration curves for specific IgE to amoxicillin by CLIA.

Spike-and-recovery tests were carried out to evaluate the differences in assay response, using representative serum samples. The results are shown in Table 1. As it can be seen, the recovery values ranged between 65 and 121%, revealing that the components in the sample matrix are not causing significant differences in assay response. The relative standard deviation values were below 10% for all dilutions.

Table 1. Recovery results obtained for moderate-level BLC-specific IgE human serum samples by CLIA.

Sample	DF[a]	Measured × DF (IU/mL)	Expected (IU/mL)	[b]R (%)
Moderate-level of BLC-specific IgE human serum	neat	1.51 ± 0.08	2.33	65
	1:3	1.98 ± 0.12		85
	1:9	2.75 ± 0.15		118
	1:27	2.82 ± 0.13		121

[a]Dilution factor. [b]Recovery. Measured values were assessed relative to the heterologous standard curve.

Clinical performances of the CLIA

A cohort of 140 sera collected from allergic patients to one or more beta-lactam antibiotics (71) and non-allergic to beta-lactam antibiotics (69) were analysed, using the developed CLIA, in triplicate for each of the selected antibiotics: penicillin G, penicillin V, piperacillin and amoxicillin; and quantified using the WHO heterologous calibration curve.

For each serum, a clinical history was available with the following information: culprit drug of the allergic episode, result of skin test of penicillin G, amoxicillin and piperacillin; and the concentration of specific IgE against penicillin G, penicillin V and amoxicillin measured by ImmunoCAP.

First, we compare the capability of each method to identify allergic and non-allergic individuals. Results that were above the detection limit (LOD) were established as positive (allergic). The LOD, defined as the lowest sIgE concentration that can be reliably determined, was 0.05 IU/mL for amoxicillin and piperacillin; and 0.06 IU/mL for penicillin G and penicillin V; being these figures below the current internationally accepted cut-off concentration for allergy diagnostics (0.35 IU/ml). For CLIA, the cut-off was 0.04 IU/mL, providing a clinical sensitivity of 64.6% and a clinical specificity of 100%.

After the analysis of the controls (non-allergic patients) it is observed that both methods identify these samples as negative, since the results obtained are below LOD of each method. The absence of false positives ratifies the good performance of the CLIA method. Table 2 lists the number of patients (allergic) analysed, showing the amount of positive (> LOD) and negative (< LOD) results obtained by both methods.

Table 2. Number of positive and negative subjects identified by CLIA and ImmunoCAP (ICAP).

Antibiotic	Allergic		Non-Allergic	
	CLIA	ICAP	CLIA	ICAP
Penicillin G	19[a] / 52[b]	7 / 64	0 / 69	0 / 69
Penicillin V	15 / 56	8 / 63	0 / 69	0 / 69
Amoxicillin	32 / 39	11 / 60	0 / 69	0 / 69
Piperacillin	18 / 53	ND	0 / 69	0 / 69

[a]positive; [b]negative; ND: not determined

As it can be inferred from the results, our CLIA system is able to detect greater number of sensitive people when analyzing samples from allergic patients, using the heterologous calibration (Table 3). Besides, the results for positive tests were compared between the two methods. In spite of being analytical systems that use different antigenic determinants, the scatter diagram (Figure 5A) shows good correlation (0.8670, $p < 0.0001$) between ImmunoCAP and CLIA, revealing the good precision of the proposed methodology. Though there are slight differences in measuring the concentration of specific IgE, CLIA performs an under-quantification of IgE, using the heterologous calibration (WHO) curve, the difference being greater at high concentrations. For amoxicillin-specific IgE, quantification was also carried out using a homologous curve. In this case, the results are similar to those obtained using the WHO curve, under-quantification of specific IgE was detected. At low IgE concentrations, the value obtained with the homology curve is very similar to the value obtained when quantifying with the WHO curve, but this behaviour changes at higher concentrations: lower values are obtained than with ImmunoCAP but higher than when using the WHO curve. This difference when using the curves (Figure 4) may be due to the matrix. In the case of the homologous curve, the matrix is raw human serum; while in the case of the heterologous (WHO) curve, the diluent media is PBS-BSA 0.1% with 0.05% Tween20. The presence of proteins in the serum causes a decrease in the signal with respect to less complex matrixes, which would explain the under-quantification when using the WHO curve.

In order to compare CLIA with the reference method at the diagnostic level, we based exclusively on the results obtained for amoxicillin, since most of the patients were clinically diagnosed as allergic to this antibiotic (47 allergic to amoxicillin). It is worth mentioning that all the positive results given by the ImmunoCAP were corroborated by the developed CLIA. The correlation between CLIA and ImmunoCAP was calculated using those samples that were positive by both methods. The analytical sensitivity of CLIA was 0.04 IU/mL. However, ImmunoCAP reports a diagnostic cut-off of 0.35 IU/mL for amoxicillin and uses a Poly-L-Lysine based antigen. All these reasons might be probably the cause of the poor correlation observed at low concentrations. Receiver Operating Characteristic (ROC) (Figure 5B) analysis showed good area under the curve for CLIA. Indeed, both *in vitro* tests showed high diagnostic specificity since no false positives were detected. Regarding diagnostic sensitivity, the CLIA classified 64.6% of patients allergic to amoxicillin as positive, compared with 23% identified by the reference test. As it can be seen, the clinical sensitivity of the developed assay was significantly better than that obtained with ImmunoCAP as reference test. Indeed, a three-fold increase of sensitivity was achieved, what confirms the strength point of the developed CLIA method.

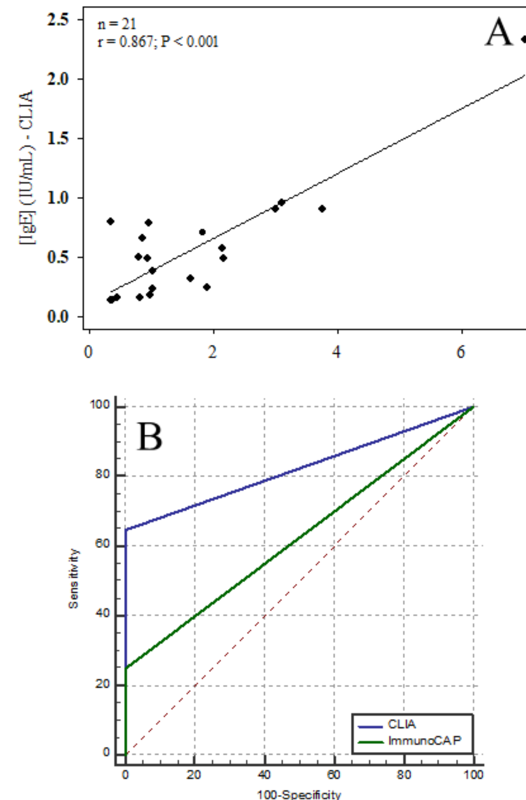

Figure 5. (A) Scatter diagram and regression line of inter-method comparison between CLIA and ImmunoCAP. (B) ROC analysis representing the area under the curve (AUC). Sensitivity and specificity of the CLIA as compared against ImmunoCAP for amoxicillin (n = 140 values).

Analysis of the results showed the high percentage of cross-reactivity that exists in BLC allergy. Of the 17 patients detected as allergic to penicillin G, none was found to be exclusively allergic to this penicillin: 88% showed cross-reactivity with amoxicillin, 47% with penicillin V and 71% with piperacillin. This is mainly because penicillin is the precursor of most β-lactam antibiotics. Of the 33 patients diagnosed as allergic to amoxicillin only 24% were allergic to this drug alone. Of the remaining, 45% had cross-reactivity with penicillin G, 33% with penicillin V and 45% with piperacillin. In the case of those diagnosed by CLIA as allergic to penicillin V, 53% presented cross-reactivity with penicillin G, 73% with amoxicillin and 67% with piperacillin. Only 13% of the patients diagnosed as allergic to penicillin V had no specific IgE capable of recognizing another of the antibiotics under study. Finally, only 6% of those diagnosed by CLIA as allergic to piperacillin were exclusively allergic to this antibiotic. Indeed, 71% of these patients

allergic to piperacillin had cross-reactivity with penicillin G, 88% with amoxicillin and 59% with penicillin V. These antibiotics are characterized by having a β-lactam ring in their molecular structure and are distinguished from each other by different side chains. Sometimes, similarity between side chains of antibiotics can lead to cross reactivity. In this process, a patient who has not been in direct contact with a certain antibiotic presents IgE capable of recognizing it. This information gives us a predictive diagnosis, which will avoid contacting the patient with an antibiotic that can trigger a high-risk allergic reaction.

Table 3. Specific IgE concentration expressed in IU/mL to β-lactams determined by CLIA and ImmunoCAP (ICAP)**.**

	Penicillin G		Amoxicillin			Penicillin V		Piperacillin
Patient	CLIA[a]	ICAP	CLIA[a]	CLIA[b]	ICAP	CLIA[a]	ICAP	CLIA[a]
1	<LOD	0.04	0.21 ± 0.01	0.20 ± 0.01	0.09	<LOD	0.01	<LOD
2	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
3	0.83 ± 0.00	13.60	1.65 ± 0.01	2.87 ± 0.01	10.20	0.40 ± 0.01	13.20	2.02 ± 0.08
4	0.22 ± 0.01	0.01	0.46 ± 0.01	0.49 ± 0.01	0.11	<LOD	0.03	0.35 ± 0.01
5	<LOD	0.00	0.11 ± 0.01	0.09 ± 0.01	0.02	<LOD	0.02	<LOD
6	<LOD	0.00	<LOD	<LOD	0.02	<LOD	0.00	<LOD
7	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.01	<LOD
8	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
9	<LOD	0.03	2.31 ± 0.01	7.02 ± 0.01	0.16	<LOD	0.00	<LOD
10	<LOD	0.00	0.05 ± 0.00	0.05 ± 0.00	0.01	<LOD	0.00	0.12 ± 0.01
11	0.49 ± 0.04	2.16	0.32 ± 0.03	0.33 ± 0.03	1.64	0.25 ± 0.02	1.91	0.47 ± 0.04
12	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
13	0.05 ± 0.01	0.00	0.16 ± 0.01	0.15 ± 0.01	0.82	<LOD	0.00	0.08 ± 0.01
14	<LOD	0.02	<LOD	<LOD	0.05	<LOD	0.03	<LOD
15	<LOD	0.03	<LOD	<LOD	0.11	<LOD	0.02	<LOD
16	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
17	<LOD	0.01	<LOD	<LOD	0.04	<LOD	0.01	<LOD
18	<LOD	0.00	0.05 ± 0.01	0.05 ± 0.01	0.01	0.08 ± 0.01	0.03	0.07 ± 0.01
19	0.16 ± 0.01	0.00	0.12 ± 0.01	0.12 ± 0.01	0.03	<LOD	0.00	0.17 ± 0.01
20	0.15 ± 0.02	0.00	0.25 ± 0.04	0.25 ± 0.04	0.01	0.07 ± 0.01	0.00	0.16 ± 0.03
21	<LOD	0.02	0.29 ± 0.03	0.29 ± 0.03	0.07	<LOD	0.02	<LOD
22	0.07 ± 0.01	0.01	0.20 ± 0.01	0.18 ± 0.01	0.04	0.07 ± 0.01	0.04	0.28 ± 0.05
23	<LOD	0.04	<LOD	<LOD	0.24	0.14 ± 0.01	0.07	<LOD
24	0.08 ± 0.01	0.01	<LOD	<LOD	0.07	<LOD	0.02	<LOD
25	0.58 ± 0.03	2.14	0.39 ± 0.02	0.41 ± 0.02	1.02	0.96 ± 0.02	3.10	0.41 ± 0.03
26	<LOD	0.00	<LOD	<LOD	0.04	<LOD	0.00	<LOD
27	0.71 ± 0.04	1.83	0.66 ± 0.04	0.76 ± 0.04	0.86	<LOD	0.97	0.13 ± 0.01
28	0.24 ± 0.02	1.02	0.50 ± 0.04	0.54 ± 0.04	0.79	0.79 ± 0.07	0.97	1.13 ± 0.09
29	0.08 ± 0.01	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
30	0.22 ± 0.01	0.08	0.17 ± 0.01	0.15 ± 0.01	0.13	0.26 ± 0.02	0.06	0.13 ± 0.01
31	<LOD	0.00	0.49 ± 0.02	0.52 ± 0.02	0.95	<LOD	0.00	<LOD
32	<LOD	0.03	<LOD	<LOD	ND	<LOD	0.03	<LOD
33	<LOD	0.00	<LOD	<LOD	0.02	<LOD	0.00	<LOD
34	1.17 ± 0.10	0.17	0.80 ± 0.07	0.96 ± 0.08	0.35	<LOD	0.10	<LOD
35	<LOD	0.02	<LOD	<LOD	0.04	<LOD	0.04	<LOD
36	<LOD	0.02	<LOD	<LOD	0.05	<LOD	0.04	<LOD
37	<LOD	0.00	<LOD	<LOD	0.02	<LOD	0.00	<LOD
38	<LOD	0.00	<LOD	<LOD	0.02	<LOD	0.00	<LOD

39	<LOD	0.01	<LOD	<LOD	0.03	<LOD	0.01	<LOD
40	<LOD	0.01	0.53 ± 0.06	0.59 ± 0.07	0.10	<LOD	0.01	<LOD
41	<LOD	0.01	0.07 ± 0.00	0.05 ± 0.00	0.03	<LOD	0.03	<LOD
42	<LOD	0.00	0.07 ± 0.00	0.06 ± 0.00	0.15	<LOD	0.03	<LOD
43	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
44	<LOD	0.12	1.38 ± 0.05	2.09 ± 0.08	0.29	0.14 ± 0.01	0.37	<LOD
45	<LOD	0.03	<LOD	<LOD	0.07	<LOD	0.15	<LOD
46	<LOD	0.01	<LOD	<LOD	0.03	<LOD	0.00	<LOD
47	<LOD	0.04	<LOD	<LOD	0.06	<LOD	0.09	0.07 ± 0.01
48	<LOD	0.01	<LOD	<LOD	0.01	<LOD	0.01	<LOD
49	<LOD	0.02	<LOD	<LOD	0.12	<LOD	0.11	<LOD
50	<LOD	0.03	0.05 ± 0.01	0.05 ± 0.01	0.08	<LOD	0.03	<LOD
51	<LOD	0.17	0.16 ± 0.04	0.15 ± 0.04	0.46	0.08 ± 0.02	0.30	<LOD
52	<LOD	0.00	<LOD	<LOD	0.02	<LOD	0.00	<LOD
53	<LOD	0.00	<LOD	<LOD	0.04	0.36 ± 0.05	0.01	<LOD
54	<LOD	0.00	<LOD	<LOD	0.03	0.40 ± 0.01	0.00	<LOD
55	<LOD	0.00	0.23 ± 0.03	0.23 ± 0.03	0.08	<LOD	0.03	<LOD
56	<LOD	0.00	<LOD	<LOD	0.03	<LOD	0.01	<LOD
57	<LOD	0.00	<LOD	<LOD	0.00	<LOD	0.00	<LOD
58	<LOD	0.00	0.17 ± 0.01	0.16 ± 0.01	0.12	<LOD	0.01	<LOD
59	<LOD	0.01	<LOD	<LOD	0.05	<LOD	0.04	<LOD
60	<LOD	0.00	<LOD	<LOD	0.09	<LOD	0.00	<LOD
61	<LOD	0.17	<LOD	<LOD	0.33	0.14 ± 0.01	0.35	0.08 ± 0.01
62	0.09 ± 0.01	0.03	0.16 ± 0.01	0.15 ± 0.01	0.24	<LOD	0.05	<LOD
63	0.08 ± 0.01	0.43	0.18 ± 0.01	0.17 ± 0.01	0.99	<LOD	0.69	<LOD
64	<LOD	0.00	<LOD	<LOD	0.00	<LOD	0.00	<LOD
65	0.91 ± 0.02	3.01	2.33 ± 0.08	7.27 ± 0.24	7.02	0.91 ± 0.03	3.76	1.21 ± 0.06
66	<LOD	0.05	0.07 ± 0.00	0.06 ± 0.00	0.41	<LOD	0.03	<LOD
67	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD
68	<LOD	0.03	0.08 ± 0.02	0.07 ± 0.01	0.24	<LOD	0.21	0.13 ± 0.01
69	<LOD	0.00	<LOD	<LOD	0.02	<LOD	0.01	<LOD
70	<LOD	0.01	<LOD	<LOD	0.05	<LOD	0.25	<LOD
71	<LOD	0.00	<LOD	<LOD	0.01	<LOD	0.00	<LOD

[a]Heterologous and [b]homologous calibration; LOD: limit of detection

CONCLUSION

The optimization of a multiparametric luminescent detection immunoassay developed in this study makes possible to determine specific IgE below 0.1 IU/mL, which allows identifying allergic patients with higher sensitivity and specificity, using only 25 µL of serum. In addition, the use of ELISA plates allows a multianalyte ELISA for screening antibiotic allergies. The use of a 96-well plate allows the analysis of specific IgE against four β-lactam antibiotics from 7 patients simultaneously and obtaining results in only one hour. In addition, CLIA is inexpensive because it does not require high-cost equipment like other diagnostic methods and allows the test to be performed with the usual material that can be found in any laboratory.

The developed CLIA has a clinical sensitivity of 64% and a specificity of 100%, making it a highly predictive assay for BLC antibiotic allergy. The detection limit set for CLIA is lower than the reference method. It might be thought that lowering the LOD would explain the increased clinical sensitivity, however there are studies that set assays with lower detection limit to those of ImmunoCAP but renders a decrease in clinical specificity[3]. A decrease in clinical specificity would result in an increase in the number of false positives, which would increase the number of people labelled as allergic to β-lactams when they are not. This incorrect labelling is an associated with negative clinical and administrative outcomes, including use of less desirable alternative antibiotics, longer hospitalizations, increasing antibiotic-resistant infections, and greater medical costs. CLIA makes it possible to increase clinical sensitivity while maintaining 100% clinical specificity, which translates into no false positives, contributing to "de-labeling", one of the main challenges in allergy diagnosis today.

On the other hand, antibiotics are the main cause of immediate perioperative hypersensitivity (POH) reactions countries as

Spain, the United States and the UK, where they represent 44%-59% of IgE-mediated POH.[23] The most frequently implicated antibiotics are β-lactams, especially amoxicillin because its widespread use. Due to the delicate state of the patients after an operation, the use of CLIA would allow a safe identification of those antibiotics that are causing the allergic reaction.

In summary, we have developed a multiparametric immunoassay with luminescent detection that can be performed in any clinical laboratory and that allows the determination of specific IgE below 0.1 IU/mL with only 25 µl of serum and one hour of testing. Furthermore, thanks to its multiparametric performance, it allows the screening of patients allergic to several antibiotics, which provides valuable information when deciding which drug to treat the patient with and defining sensitization profiles. The clinical performance is good and represents a significant improvement in the clinical sensitivity of the most currently used methods for the diagnosis of BLC antibiotic allergy. Consequently, CLIA allows the diagnosis of allergy to penicillin G, penicillin V, amoxicillin and piperacillin with a high predictive value, in a cheap, fast and simple way.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Blocking step study, coating amount of H1-BLcs antigen study, sample dilution study and interactive dot diagram.

AUTHOR INFORMATION

Corresponding Author

*Sergi Morais - IDM, Universitat Politècnica de València-Universitat de València, Camino de Vera s/n, (46022) Valencia, Spain; E-mail: smorais@upv.es

Author Contributions

P.Q.C. and S.M. designed the experiments; P.Q.C. and M.J.J. performed the experiments, and S.M. supervised the work. P.Q.C., S.M. and A.M. wrote and revised the manuscript. All authors have given approval of the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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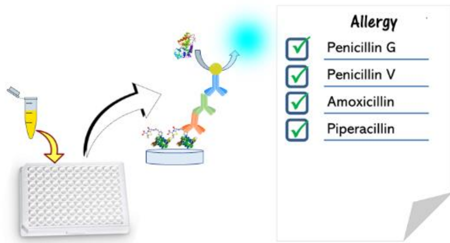

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