An ultra-sensitive homologous chemiluminescence immunoassay to tackle penicillin allergy

Pedro Quintero-Campos[1], Paula Segovia-de los Santos[2], Ethel Ibáñez-Echevarria[3], Dolores Hernández-Fernández de Rojas[4], Patricia Casino[5,6,7], Gabriel Lassabe[2], Gualberto González-Sapienza[2], Ángel Maquieira[1,8,9] Sergi Morais*[1,8,9]

[1]*Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València-Universitat de València, Spain.*

[2]*Cátedra de Inmunología, Facultad de Química, DEPBIO, Instituto de Higiene, Montevideo, Uruguay.*

[3]*Hospital Universitari I Politènic La Fe, Servicio de Alergia,*

[4]*Allergy Therapeutics Ibérica. Av. de Barcelona, 115, 08970, Sant Joan Despí, Spain.*

[5]*Departamento de Bioquímica y Biología Molecular,* [6]*Instituto Universitario de Biotecnología i Biomedicina (BIOTECMED), Universitat de València. Dr Moliner 50, 46100, Burjassot, Spain.*

[7]*Group 739 of the Centro de Investigación Biomédica en Red sobre Enfermedades Raras (CIBERER) del Instituto de Salud Carlos III, Spain.*

[8]*Unidad Mixta UPV-La Fe, Nanomedicine and Sensors, IIS La Fe, Av. de Fernando Abril Martorell, 106, 46026 València, Spain.*

[9]*Departamento de Química, Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain.*

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

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ABSTRACT

Penicillin is one of the most widely used antibiotics to treat bacterial infections in clinical practice. The antibiotic undergoes degradation under physiological conditions to produce reactive compounds that *in vivo* bind self-proteins. These conjugates might elicit an immune response and trigger allergic reactions challenging to diagnose due to the complex immunogenicity. Penicillin allergy delabeling initiatives are now part of antibiotic stewardship programs and include the use of invasive and risky *in vivo* tests. Instead, the *in vitro* quantification of specific IgE is highly useful to confirm immediate allergy to penicillins. However, discrepant results associated with the low sensitivity and accuracy of penicillin allergy *in vitro* tests have limited their routine diagnostic use for delabeling purposes. We aimed to develop a homologous chemiluminescence-based immunochemical method for the reliable determination of specific IgE to penicillin G, using unprecedented synthetic human-like standards. The synthetic standard targets the major antigenic determinant of penicillin G and the paratope of Omalizumab, acting as human-like specific IgE. It is a potent calibrator, highly stable, easy, and inexpensive to produce, overcoming the limitations of the pooled human serum preparations. The developed method achieved a good agreement and strong positive relationship, reaching a detection limit below 0.1 IU mL^{-1} and excellent reproducibility (RSD < 9 %). The clinical sensitivity of the assay significantly increased (66 %), doubling the accuracy of the reference method with an overall specificity of 100 %. The new diagnostic strategy compares favorably with results obtained by the standard procedure, paving the way towards the standardization of penicillin allergy testing and enhancing the detection sensitivity of specific IgE in serum to tackle reliably β-lactam allergy delabeling.

Keywords: allergy, binanobody, diagnostics, Omalizumab, antibiotics

Abbreviations:

- BSA: Bovine serum albumin
- Fab region: Fragment antigen-binding
- Fc region: Fragment crystallizable region
- H1: Histone H1
- hmCLIA: Homologous Chemiluminescence Immunoassay
- HRP: Horseradish Peroxidase
- HSA: Human Serum Albumin
- IgE: Immunoglobulin type E
- IPTG: Isopropyl β-D-1-thio-galactopyranoside
- IU: International Units
- KLH: Keyhole limpet hemocyanin
- LOD: Limit of detection
- LOQ: Limit of quantification
- PEG: Polyethylene glycol 800
- PG: Penicillin G
- PSU: Penicillin Standardised Unit
- rBBN: Recombinant bispecific binanobody
- SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
- sIgE: Specific IgE
- TMB: 3,3′,5,5′- tetramethylbenzidine
- VHH: Antigen binding fragment of heavy chain only antibodies
- WHO: World Health Organization

1. INTRODUCTION

Penicillin G is a broad-spectrum β-lactam antibiotic that is the primary choice for treating numerous bacterial infections [1, 2]. The antibiotic itself is not immunogenic due to its small size but undergoes degradation to produce reactive compounds that bind *in vivo* intra or extracellular proteins under physiological conditions [3, 4]. These conjugates, classified as major or minor determinants relative to the quantity produced, may elicit an immune response, resulting in allergic reactions [5]. The procedure *in vivo* is complex; however, it has been possible to identify the major determinant based on penicilloyl groups [6] and use it for *in vitro* allergy diagnosis.

In Europe, Northern America, and Australia, allergy to penicillins is reported by up to 10 % of the general population and 15 % of hospitalized patients [7]. Consequently, this contributes to the prescription of antibiotics that diverge from the guidelines, increasing antimicrobial resistance, one of the biggest threats to global health. However, a vast majority of these patients would be classified as β-lactam tolerant upon appropriate assessment by an allergist.

Delabeling initiatives are now part of antibiotic stewardship programs [8] and include the use of invasive, time-consuming, and risky *in vivo* tests. Instead, the quantification of specific IgE to penicillins is highly valuable for allergy monitoring, confirming or ruling out immediate allergy, and finding safe alternatives. However, the current *in vitro* tests lack sensitivity and accuracy (ca. 81% false negatives), limiting their routine diagnostic use for delabeling purposes [7, 9].

In vitro diagnosis of human allergies is based on quantifying specific IgE (sIgE) in serum [10]. Currently, several assays are commercially available for the determination of sIgE, such as ImmunoCAP (Thermo Fisher Scientific), Immulite and Atellica (Siemens), Euroline (Euroimmun), Noveos (Hycor), ALEX2 (Macro Array Diagnostics) and ALFA (Dr. Fooke). They are all based on the same principle, but differ in the degree of automation, allergen binding way, signal detection mode, required sample volume, and type of analysis [11]. Yet, these methods have a detection limit above 0.1 IU mL-1 and low accuracy in correctly identifying patients allergic to penicillins [7, 12]. The poor clinical performances of the *in vitro* assays in use for penicillin allergy diagnostic testing can be attributed to the low selectivity of antigens, the type of calibration designed, the detection system, and the low analytical sensitivity of the methods [13, 14]. The paramount unmet need for *in vitro* assays for the diagnosis of penicillin allergy is large-scale

validation studies with standardized controls that could harmonize diagnostic management between the European Union, United States, Australia, and other regions of the world.

We recently introduced a multiparametric heterologous chemiluminescent immunoassay with improved clinical performances [15] by using a nuclear antigen and luminescent detection. However, as with other quantitative immunoassays, results are not comparable with different methodologies due to the lack of standardization [11]. Accordingly, there is an urgent need to harmonize reagents, methods, and protocols for the *in vitro* diagnosis of drug allergy.

The lack of standardized allergen-specific IgE reference preparations is one of the weaknesses of *in vitro* diagnosis [16]. Concentrations of sIgE are calculated from a total IgE calibration curve by a heterologous interpolation. A set of calibrators (WHO 11/234) is used to transform the assay signals into quantitative allergen-specific IgE antibody units (IU mL-1) [17]. However, recent studies have revealed a better correlation among methods when homologous calibration is used for quantifying allergen-specific IgE [13, 15]. Therefore, the absence of internationally standardized allergen-specific IgE reference preparations is one of the reasons for the discrepant results between methods and probably the most critical unresolved issue to harmonize *in vitro* serological quantitative allergy diagnostics.

The standard for the homologous calibration is the sIgE present in serum. Serum samples, however, show variability in terms of sIgE level, the concentration of β-lactam antibiotics being very low[18]. Furthermore, the sample volume is limited, and its accessibility is difficult and expensive. Therefore, standardized reference material is currently not available. Besides, positive human sera are crucial to performing the so-called "round-robin" tests to evaluate the accuracy and precision of the *in vitro* allergy tests between laboratories.

The first attempt approaching synthetic standards consisted of raising specific antibody molecules by hyperimmunization to produce a fully-humanized chimera [19]. Later, these molecules were produced recombinantly by binding the Fab to the human Fc region, demonstrating the ability to bind target ligands and displaying stability for producing therapeutic molecules [20, 21].

An advantageous alternative to conventional antibody-based standards is the use of nanobodies. These are recombinant antibody fragments (15 kDa) derived from the variable domain (VHH) of a particular type of antibody found in camelids devoid of light chains[22]. They have

remarkable physicochemical properties, including thermal stability and solubility, and they are produced with high yields by bacterial fermentation. In addition, their monodomain nature facilitates the construction of phage display libraries, which offers the possibility of isolating nanobodies with the desired properties [22].

We recently presented the generation and characterization of standardized synthetic standards consisting of a nanobody against the paratope of the detection antibody expressed in tandem with a second nanobody against the target antigen for immunodiagnosis [23].

This study presents the development of an accurate homologous chemiluminescence immunoassay to assess the potential analytical value of synthetically produced human IgE-like standards. The synthetic molecules were used as calibrators for the selective quantification of specific IgE to penicillin G, the most used β-lactam antibiotic [24], at low levels (< 0.1 IU mL-1). The developed immunochemical approach is evaluated by analyzing a cohort of 65 human serum samples.

2. MATERIALS AND METHODS

2.1. Reagents, buffers, and consumables

Keyhole limpet hemocyanin (KLH), human serum albumin (HSA), histone H1, penicillin G (PG), bovine serum albumin (BSA), D-biotin, isopropyl β-D-1-thio-galactopyranoside (IPTG), polyethylene glycol 8000 (PEG), LB Broth (Miller), 3,3′,5,5′- tetramethylbenzidine (TMB), tris base, Tween 20 and other chemicals were from Sigma (St. Louis, MO, USA).

Anti-hemagglutinin epitope (anti-HA) antibody conjugated to horseradish peroxidase (HRP) was from Sigma (Cat no. 12013819001). Omalizumab was from Novartis International AG (Basel, Switzerland). Anti-human IgG antibody-HRP (Cat No. ab97225) and the HRP Conjugation Kit (ab102890), the latter used to prepare Omalizumab-HRP, were from Abcam (Cambridge, UK).

E. coli One Shot BL21 (DE3) cells were from Thermo Fisher Scientific (Fremont, CA, USA). *E. coli* ER2738 electrocompetent cells were purchased from Lucigen Corporation (Middleton, WI, USA), and chromatography columns were from GE Healthcare (Piscataway, NJ, USA). High binding polystyrene ELISA plates were from Costar Corporation (Cambridge, MA, USA), SuperSignal ELISA Femto Substrate, PCR Plate 96-well, and SYPR Orange Protein Gel Stain were 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).

2.2. Production of antigens

Penicillin G-derived antigens were prepared as follows [25]. The corresponding amount of penicillin G sodium salt (98%, w/w) was reacted with the carrier molecules (5.0 mg; molar ratio 1:1000) dissolved in 1.0 mL of 0.5 M sodium carbonate pH 11.0 overnight at room temperature. HSA, KLH, and H1 were the carrier proteins to prepare the PG-derived antigens for the immunization, panning, and analysis of serum samples. The antigens were purified using centrifugal filters (Amicon Ultra centrifugal filters) and PBS as buffer exchange, and the concentration was determined with the Bradford protein assay. All the antigens were characterized by SDS-PAGE electrophoresis (Supporting information).

2.3. Phage Display

An adult llama (*Lama glama*) from the municipal zoo of Montevideo was immunized subcutaneously with 4 doses of 500 µg of HSA-PG conjugate in Freund's Incomplete Adjuvant. Ten days after the last immunization, 200 mL of blood was drawn. From the extracted blood, lymphocytes were purified by gradient centrifugation with Histopaque (Sigma) according to the manufacturer's protocol. From 10^8 cells, total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA, and retrotranscribed to cDNA using the RevertAID Reverse Transcriptase (Thermo Fisher Scientific, Carlsbad, CA, USA). Subsequently, DNA fragments encoding the VH/VHH of the IgGs were amplified by PCR as described in the supporting information. Fragments obtained were digested using SfiI, cloned into the pComb3X phagemid vector (from Dr. Barbas, The Scripps Research Institute, La Jolla, USA), and electroporated into *E. coli* ER2738 competent cells. Finally, cells were cultured and superinfected with the helper phage M13KO7 to generate the phage library. Next, two rounds of panning were performed. The eluted phages were used to infect ER2738 cells and grown on LB/agar plates. 2 mL cultures of each clone were grown in SB with 0.1 mg mL-1 ampicillin at 37 °C and shaking from the isolated bacterial colonies until an optical density of 0.6 was reached. After that, nanobody expression was induced by adding 1 mM IPTG and incubated with shaking at 37 °C for 16 hours. After centrifugation, supernatants

containing the nanobodies were collected and tested in ELISA plates coated with KLH-PG conjugate. Bound nanobodies were detected with an anti-HA-HRP conjugate following the protocol described in the supporting information.

2.4. Cloning, expression, and purification of recombinant bispecific binanobody

Clones specific to the PG antigen were selected, and the VHH genes were amplified and subcloned into the pINQ-bis vector. This vector is an in-house modified version of the pET-28a(+) vector that allows the expression of bispecific nanobodies in the periplasm of E. coli BL21(DE3). The modification resulted in an expression cassette coding for ompA signal peptide, the selected anti-β-lactam VHH flanked by two SfiI restriction sites, and a spacer of GQAGR(GGGGS)3TSEL, followed by an anti-omalizumab VHH sequence and a 6xHis tag. The vector was transformed into *E. coli* strain (DE3). Individual clones isolated on LB-kanamycin plates were grown in 500 mL of LB with kanamycin (50 μg mL^{-1}) and induced with 10 μM IPTG for 16 hours at 28 °C under shaking conditions.

According to previously established protocols, cultures were centrifuged, and periplasmic proteins were extracted by osmotic shock [26]. Then, the recombinant bispecific binanobody (rwas were purified on 1 mL His-Trap columns on the ÄKTA purification system (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The imidazole-eluted fractions were dialyzed against PBS and quantified spectrophotometrically (Abs 280 nm 0.1% = 1.9) and by Bradford's protein assay.

2.5. Biochemical characterization of rBBN

The purified rBBN was analyzed by 12 % sodium ddodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining method. The specificity and functionality of the rBBN were evaluated by ELISA, blue native PAGE, and ThermoFluor assay as described in the supporting information.

2.6. Analysis of serum samples

Serum samples from 65 adults were collected in red-top tubes (BD Diagnostics, Madrid, Spain) and 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 -80 ºC until use. A cohort of 35 allergic patients who reported a confirmed history of penicillin allergy and 30 non-allergic individuals were included in the case-control study. Clinical history included the concentration of sIgE measured by the ImmunoCAP system.

This study was approved by the Hospital Universitari i Politècnic La Fe (Valencia, Spain) ethical review committee. All experiments were performed following the relevant guidelines and regulations.

Samples were analyzed by the homologous chemiluminescence immunoassay (hmCLIA). Briefly, a white flat-bottomed polystyrene ELISA plate was coated with H1-PG conjugate [27] solution (3 μg mL^{-1}) in coating buffer. The next day, the plate was washed 4 times with PBS-T. After that, 25 μL of serum sample was added to each well, followed by incubation for 30 min at room temperature. The calibration curve was made using the synthetic standards diluted in a pooled control sIgE-free human serum. All serum samples were analyzed in triplicate. Subsequently, after washing the plate, 25 μL/well of Omalizumab-HRP solution (1/10,000 dilution) was incubated. After, 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 EnSpire Multimode Plate Reader (PerkinElmer, Waltham, USA). The limit of detection (LOD) and quantification (LOQ) were calculated by measuring ten times the blank (pooled negative controls) and interpolating the mean signal plus 3 and 10 times the standard deviation to the calibration curve respectively. Specific IgE levels expressed in PSU mL^{-1} (Penicillin Standardised Unit) were determined by interpolating the luminescent signals into the calibration curve.

2.7. Statistical analysis

Coefficients of variation and the parameters to evaluate the assay sensitivity were calculated 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 the reference method.

3. RESULTS AND DISCUSSION

3.1. Phage display

The antibody titer of the llama serum (Figure S2) was high after the fourth boost, revealing the production of specific antibodies to PG. Next, a phage-display VHH library with a size of 1 × 108 transformants was constructed from the lymphocytes of the immunized llama (see supporting information). Ten individual clones were selected, and their reactivity was tested against a panel of antigen determinants to β-lactam antibiotics. As is shown in Figure S3, seven out of ten clones were specific to penicillin G (PG1-5, PG7-8), two clones were generic to the panel of determinants (PG9-10), and one was a nonspecific clone (PG6) as it showed reactivity against the carrier protein KLH. The PG8 clone, the best in performance, was selected for further cloning and expression.

3.2. Cloning, expression, and purification of rBBN

After subcloning into the pINQ-bis vector [23], sequencing confirmed that the clones possessed the two VHHs sequences of interest. Figure 1A depicts the structural model of rBBN using the Robetta web server and visualized in UCSF Chimera. The model shows the two nanobodies connected by a loop (yellow); the 17 kDa one (blue) targets the penicillin G determinant, and the 18 kDa one (green) the paratope of Omalizumab. Purified rBBN was analyzed by 12 % SDS-PAGE gel electrophoresis. A clear and intense band (lane 3, Figure 1B) was observed, corresponding to the synthetic standard with an expected molecular weight of around 35 kDa.

3.3. Biochemical characterization of rBBN

The functionality of rBBN was tested by ELISA, following the protocol described in the supporting information. As shown in Figure 2A, the set of data fitted well to a four-parameter logistic model, revealing the standard was functional, as it targeted both the antigenic determinant to penicillin G and the paratope of Omalizumab. According to the lowest detectable concentration, the limit of detection was 0.13 ng mL^{-1} (3.7 pM) under the ELISA conditions.

We also conducted a thermofluor assay to check if the interaction of rBBN with H1-PG stabilized the complex. As shown in Figure 2B, the Tm of rBBN alone is 59 °C, while the Tm of rBBN in the presence of H1-PG increased around 7 °C. This result indicates that the rBBN-Penicillin G complex is more stable than rBBN alone, demonstrating the specific binding between the nanobody and the ligand.

The selectivity of rBBN for H1-PG and Omalizumab was also studied by Blue Native-PAGE, as rBBN has a high isoelectric point (pI = 8.5) which prevented the protein from entering the gel in a regular Native-PAGE. In this way, tested proteins were mixed with a loading buffer containing Coomassie Blue G (Serva, GmbH), which adds negative charges to proteins and forces them to enter the gel and run according to their molecular weight (MW) and hydrodynamic conformation. Figure 2C shows the electrophoretic mobility of the proteins alone, and that of the complex. The rBBN at 1 and 3 μM ran as a single band with high MW (~245 kDa), possibly due to the high hydrodynamic volume caused by the conformational flexibility of the loop connecting the two nanobodies. In the case of the H1-PG conjugate, three bands were shown, one at ~55 kDa, which we identified as the H1-PG conjugate monomer, a faint band around ~100 kDa, which could be ascribed to a dimer, and another at high MW (~ 245 kDa) ascribed to multimers. Then, rBBN and H1-PG were mixed at a molar ratio of 1:1 (lane 4) and 1:3 (lane 5) for 15 minutes. A decrease in the intensity for the 55 kDa band of H1-PG was observed as the amount of rBBN increased.

In contrast, protein accumulation around the higher MW band was shown, thus, ascribing the electrophoretic mobility changes to the complex formation. When Omalizumab was assayed, it barely entered the gel producing two faint bands at ~150 kDa and at ~245 kDa, which could be ascribed to the monomeric and dimeric antibody, respectively and the protein accumulation at the top of the gel that was ascribed to multimers. The addition of rBBN to Omalizumab was concomitant with the disappearance of the 150 kDa band and a decreased intensity for the accumulated band, which indicated complex formation. Finally, incubation of the three samples, H1-PG/rBBN/Omalizumab, was performed at a molar ratio of 1:1:1 and 1:3:1. As observed before, the band of 150 kDa corresponding to H1-PG disappeared, and a protein accumulation for the high MW band (245 kDa) was shown. Interestingly, the intensity of the Omalizumab band at the top of the gel dramatically decreased. These changes in electrophoretic mobility are indicative of complex formation.

3.4. Analysis of serum samples

The produced rBBN was used as a standard to determine the concentration of sIgE to penicillin G present in a representative serum sample, following the homologous calibration design (Figure 3A) in the hmCLIA method. The current method for the determination of sIgE follows a heterologous calibration and defines 1.0 IU mL^{-1} is equivalent to 2.4 ng mL^{-1} IgE. Due to the remarkable difference in molecular weight between the international standard IgE (190 kDa) and rBBN (35 kDa), a new unit (Penicillin Standardised Unit, PSU), equivalent to 0.44 ng mL^{-1} of rBBN was established based on the IgE-rBBN molar ratio which is 5.4. To build the calibration curve, rBBN was diluted in sIgE-free serum at concentrations of 95, 54, 19, 5.4, 3.8, 1.90, and 0.5 PSU mL^{-1}. The stability of rBBN was studied for a period of 8 h. The results revealed that there were no statistically significant differences ($p < 0.0001$) between the signals obtained over the time (1-8 h), which guarantees its good performance during the total assay time.

Figure 3B shows the calibration curve. The signals ($n = 10$) were fitted to a four-parameter logistic curve, giving a dynamic response ranging from 0.1 to 95 PSU mL^{-1} and a LOD of 0.02 PSU mL^{-1} (8.8 pg mL^{-1}), a concentration below the current internationally accepted cut-off for allergy diagnosis (0.1 IU mL^{-1} or 240 pg mL^{-1}). In the hmCLIA accuracy assessment, the CVs rarangedrom 2 to 9%, resulting in a linear regression equation with a correlation coefficient (r) of 1. Therefore, the results revealed that the hmCLIA assay, using the new standards under a homologous calibration, has good analytical performances.

A cohort of 65 sera collected from patients allergic to penicillin G (35) ,and non-allergic subjects (30) was analyzed in triplicate by hmCLIA assay. The signals obtained for each sample were interpolated in both the homologous (synthetic standard) and heterologous (WHO international standard) calibration curves. Table 1 shows the results. The Pearson's correlation coefficient statistical study (Figure 4A) displays that there is a direct positive correlation ($r = 0.995$, $p < 0.0001$) between the two calibration designs. Furthermore, the results obtained under the homologous calibration relate well with those obtained by ImmunoCAP, showing a Pearson correlation close to 1 ($r = 0.999$, $p < 0.0001$) (Figure 4B). In addition, as shown in Figure 4B, the

slope of the curve is 3, representing the relationship between the value given by ImmunoCAP and that obtained using rBBN as standard.

The concentrations given by the hmCLIA assay are multiplied by a factor of 1/3 to get those obtained by ImmunoCAP (see results in Table 1). This factor allows the classification of patients in the same RAST group. The new standard results also showed recovery values ranging from 91 to 135%, revealing a reliable quantification compared with the reference approach.

The linearity of dilution test was performed using a pooled positive serum sample (sample 36 in Table 1). The linearity dilution study was carried out using 4-fold serial dilutions (1/4-1/4096) prepared in the pooled control serum. ImmunoCAP and hmCLIA calculated the experimental concentrations. As shown in Figure 4C, the linearity of the hmCLIA assay was good over a wide range of dilution, detecting sIgE up to the dilution 1/1024 (0.04 PSU mL-1) revealed good flexibility to quantify low levels of sIgE.

On the other hand, we compared the ability of our assay to identify allergic and non-allergic individuals. Analysis of the controls (non-allergic patients) shows that hmCLIA, using the standards identifies all the control samples as negative, as the results obtained are below the LOD. The absence of false positives reveals the excellent specificity (100%) of the hmCLIA method.

Regarding the sensitivity, the hmCLIA method identified 23 out of the 35 positive samples (66%), while ImmunoCAP detected 10 (28%) positive samples. To compare hmCLIA with the reference method at the diagnostic level, the correlation was made using the samples detected as positive by both assays. It is worth mentioning that all the positive results given by the ImmunoCAP were also positive by hmCLIA. Receiver Operating Characteristic (ROC) (Figure 4D) analysis showed a good area under the curve for hmCLIA. In summary, the clinical sensitivity of the developed assay was significantly better, doubling that of the ImmunoCAP assay, which allowed the identification of more positive allergic patients.

4. CONCLUSIONS

Here we present a highly sensitive homologous immunochemical approach that uses selective bispecific binanobodies as synthetic standards to quantify specific IgE, improving the performances of current *in vitro* tests for penicillin G allergy testing. This strategy might contribute to the harmonization of this diagnosis and facilitate the appropriate delabeling of patients

categorized as allergic to penicillin, thereby reducing the spread of antibiotic resistance and healthcare costs and optimizing patient outcomes. The synthetic standard is robust, easy, and inexpensive to produce in any laboratory with identical characteristics requiring only their sequence. This calibrator circumvents the issues of availability and reproducibility related to the use of pooled human sera and defibrinated plasma and avoids potential risks of transmission of infectious diseases. This positions the homologous chemiluminescence immunoassay as a promising immunochemical approach for inter-laboratory comparison studies, allowing verification of whether the methods produce results that agree with the reference one, providing the basis for certificates of quantitative analysis.

In summary, we truly believe that the work presented here represents a significant step toward the standardization of *in vitro* tests to determine allergen-specific IgE antibodies. This might support the reliable quantification of the level of sIgE directly to establish a more precise RAST classification. In addition, producing this generation of calibrators with different specificities would allow more precise diagnostics to successfully tackle drug allergy delabeling to other antibiotics.

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Table 1: Specific IgE to Penicillin G determined by hmCLIA and ImmunoCAP (ICAP).

	Calibration				
	WHO	WHO	Binanobody		
Patient	**ICAP (IU mL^{-1})**	**CLIA (IU mL^{-1})**	**hmCLIA (PSU mL^{-1})**	**hmCLIA* (PSU mL^{-1})**	**Recovery (%)**
1	< 0.1	0.6 ± 0.1	7.1 ± 0.4	2.4 ± 0.1	--
2	3.5	0.7 ± 0.1	9.5 ± 0.8	3.2 ± 0.3	91
3	< 0.1	< 0.1	< 0.02	< 0.02	--
4	13.6	2.5 ± 2.5	43.1 ± 0.7	14.4 ± 0.2	106
5	< 0.1	< 0.1	< 0.02	< 0.02	--
6	< 0.1	< 0.1	< 0.02	< 0.02	--
7	< 0.1	0.9 ± 0.1	12.6 ± 0.8	4.2 ± 0.3	--
8	< 0.1	0.3 ± 0.1	3.7 ± 0.1	1.2 ± 0.1	--
9	2.2	0.9 ± 0.1	9.7 ± 0.6	3.2 ± 0.2	148
10	< 0.1	< 0.1	< 0.02	< 0.02	--
11	< 0.1	< 0.1	< 0.02	< 0.02	--
12	< 0.1	2.7 ± 0.1	47.3 ± 0.4	15.8 ± 0.2	--
13	10.0	1.9 ± 0.2	32.4 ± 3.0	10.8 ± 1.0	108
14	< 0.1	0.8 ± 0.1	11.7 ± 0.2	3.9 ± 0.1	--
15	< 0.1	< 0.1	< 0.02	< 0.02	--
16	1.8	0.5 ± 0.1	6.5 ± 0.4	2.2 ± 0.1	118
17	< 0.1	0.6 ± 0.2	8.1 ± 0.2	2.7 ± 0.1	--
18	< 0.1	0.6 ± 0.1	7.4 ± 0.1	2.5 ± 0.1	--
19	< 0.1	0.3 ± 0.1	3.2 ± 0.2	1.1 ± 0.1	--
20	1.8	0.5 ± 0.1	6.0 ± 0.2	2.0 ± 0.1	109
21	2.2	0.5 ± 0.1	6.7 ± 0.4	2.2 ± 0.1	112
22	< 0.1	< 0.1	< 0.02	< 0.02	--
23	< 0.1	0.9 ± 0.1	13.2 ± 0.1	4.4 ± 0.1	--
24	< 0.1	0.3 ± 0.1	3.6 ± 0.3	1.2 ± 0.1	--
25	< 0.1	< 0.1	< 0.02	< 0.02	--
26	< 0.1	< 0.1	< 0.02	< 0.02	--
27	0.4	0.2 ± 0.1	1.2 ± 0.1	0.4 ± 0.1	92
28	3.0	1.0 ± 0.1	12.2 ± 0.4	4.1 ± 0.1	136
29	< 0.1	< 0.1	< 0.02	< 0.02	--
30	< 0.1	1.3 ± 0.1	21.0 ± 0.2	7.0 ± 0.1	--
31	< 0.1	< 0.1	< 0.02	< 0.02	--
32	< 0.1	< 0.1	< 0.02	< 0.02	--
33	91.1	40.9 ± 0.9	270.5 ± 6.0	90.2 ± 2.0	99
34	< 0.1	0.6 ± 0.1	8.0 ± 0.5	2.7 ± 0.2	--
35	< 0.1	0.5 ± 0.1	5.3 ± 0.1	1.8 ± 0.1	--
36[a]	12.5	4.5 ± 0.4	41.1 ± 3.8	13.3 ± 1.1	106

*The concentrations are multiplied by a factor of 1/3 to get those obtained by ImmunoCAP. [a]Pooled-sample from patients 2, 4, 9, 13, 16, 20, 21, 27, 28 and 33, using equal volume of each.

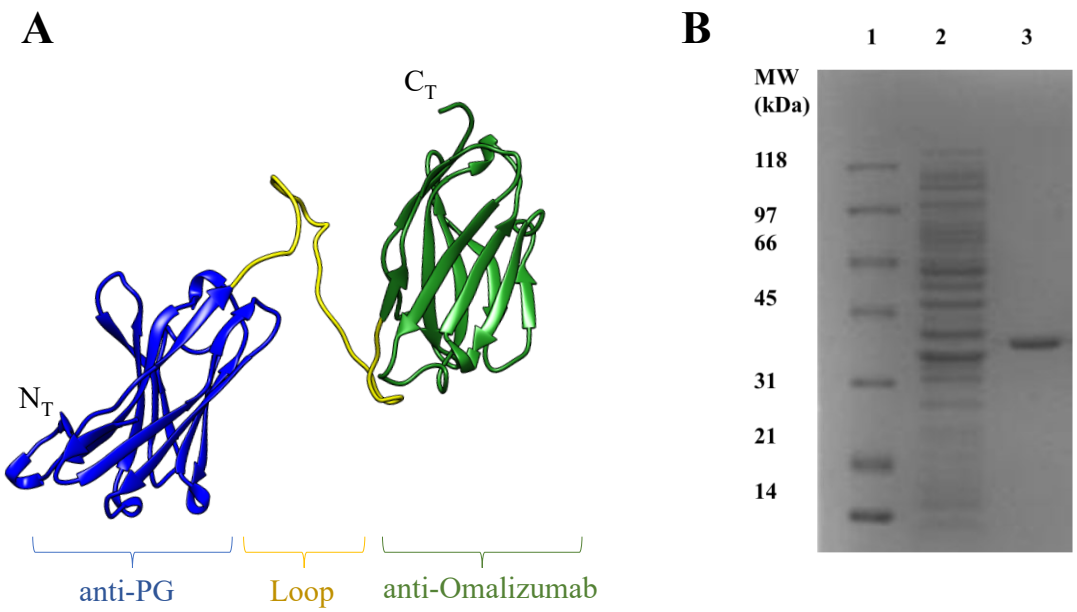

Figure 1. [A] Model structure of the rBBN. On the left (blue), the nanobody that targets the antigenic determinant, on the right (green) the one that binds the paratope of Omalizumab. **[B]** Purification results 12 % SDS-PAGE: (1) Ladder (2) Purification Flow Through (3) Bispecific binanobody with a molecular weight of 35 kDa.

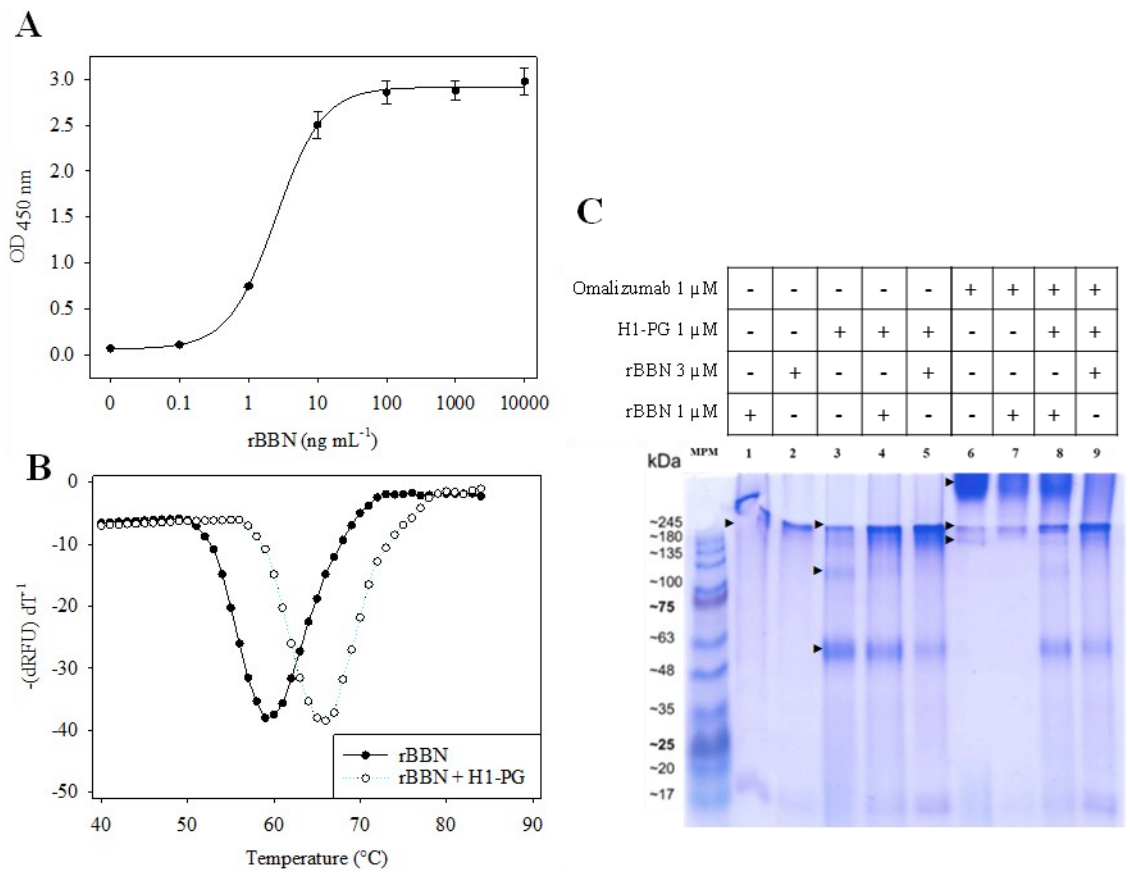

Figure 2. [A] Curve of functionality of the bispecific binanobody. **[B]** Results of thermofluor assay for rBBN alone and bound to conjugate H1-PG. Representation of the melting curve using the first derivative –(dRFU)/dT of the raw data. The Tm is the apex**. [C]** Blue Native Page: (1) rBBN 1 µM. (2) rBBN 3 µM. (3) H1-PG 1 µM. (4) H1-PG 1 µM + rBBN 1 µM. (5) H1-PG 1 µM + rBBN3 µM. (6) Omalizumab 1 µM. (7) rBBN 1 µM + Omalizumab 1 µM. (8) H1-PG 1 µM + rBBN 1 µM + Omalizumab 1 µM. (9) H1-PG 1 µM + rBBN 3 µM + Omalizumab 1 µM**.**

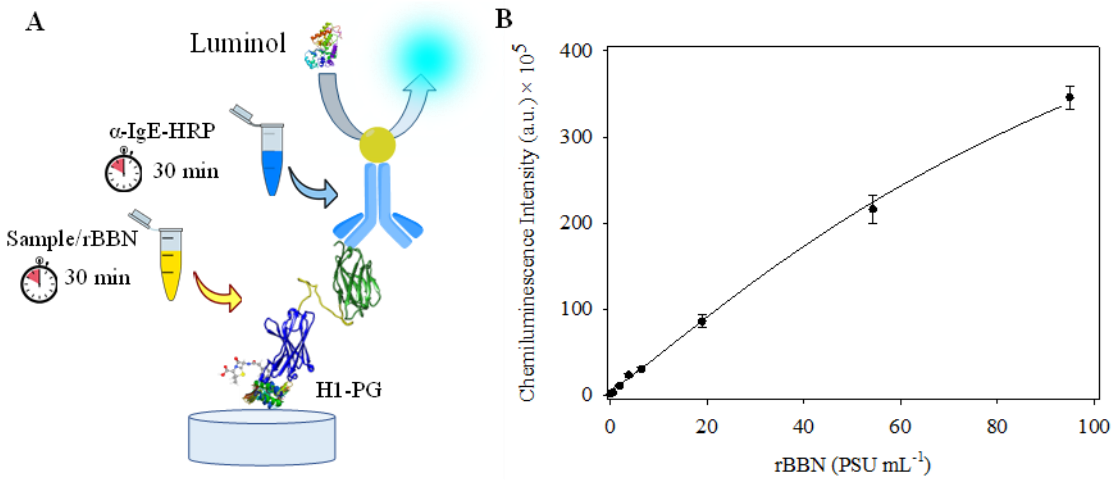

Figure 3. [A] Scheme of the homologous chemiluminescence immunoassay for the determination of specific IgE to penicillin G in human serum, using the rBBN as calibrator. **[B]** Binanobody calibration curve.

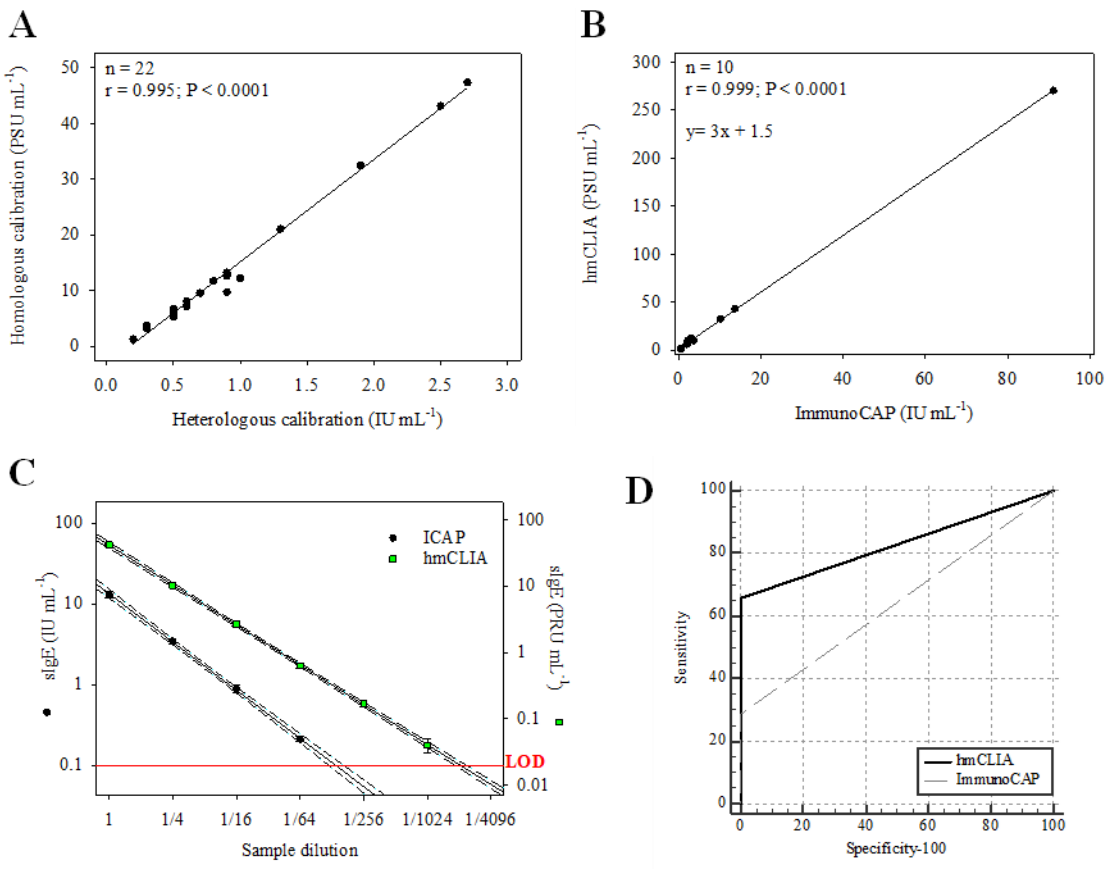

Figure 4: **[A]** Scatter diagram and regression line of inter-method comparison between homologous calibration (rBBN) and heterologous calibration (WHO). **[B]** Scatter diagram and regression line of inter-method comparison between hmCLIA and ImmunoCAP. **[C]** Dilution linearity study, using the pooled serum sample and a pool of control serum samples as diluent. Dotted lines represent the 99% confidence interval. **[D]** ROC analysis representing the area under the curve (AUC). Sensitivity and specificity of the hmCLIA as compared against ImmunoCAP for penicillin g (n = 65 values).