INSTITUTO DE RECONOCIMIENTO MOLECULAR Y DESARROLLO TECNOLÓGICO

TESIS DOCTORAL

Diseño, obtención y caracterización de proteínas recombinantes a partir de determinantes antigénicos asociadas a reacciones alérgicas a β-lactámicos.

Presentada por Pedro Quintero Campos para optar al grado de Doctor por la Universitat Politècnica de València

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València, Diciembre 2022

A mis padres, Soledad y José Pedro

«La ciencia es respecto del alma lo que es
la luz respecto de los ojos, y si las raíces
son amargas, los frutos son muy dulces.»

Aristóteles

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Que el trabajo que presenta Pedro Quintero Campos en esta memoria, con título "Diseño, obtención y caracterización de proteínas recombinantes a partir de determinantes antigénicos asociadas a reacciones alérgicas a β-lactámicos" ha sido realizado bajo nuestra dirección en el Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM) para optar al grado de Doctor por la Universitat Politècnica de València.

Para que así conste, firman el presente certificado en Valencia, 15 de diciembre de 2022.

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Montevideo, 12 de diciembre de 2019

A quien corresponda

Por la presente dejo constancia que el Lic. Pedro Quintero ha realizado una estadía de investigación en mi laboratorio, desde el 14 de Setiembre al 18 de diciembre de 2019, trabajando en la selección y generación anticuerpos monodominio de camélidos.

Sin otro particular, saluda atte.

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RESUMEN

Los antibióticos β-lactámicos son los fármacos más utilizados en todo el mundo para tratar infecciones bacterianas, debido a su eficacia, seguridad y coste, representando el 65% del mercado mundial de antibióticos. Sin embargo, estos fármacos, entre los que se encuentran la penicilina y todos sus derivados, son capaces de generar una respuesta alérgica, siendo la causa más común de reacciones farmacológicas inmunomediadas. De hecho, hasta un 10% de la población general declara ser alérgica a alguno de estos antibióticos. No obstante, un alto porcentaje de los pacientes etiquetados como alérgicos serían clasificados como tolerantes tras una evaluación clínica precisa. Es por eso por lo que las iniciativas de desetiquetado de los pacientes mal catalogados son ahora parte de los programas de administración de estos fármacos. Así, se evita una clasificación errónea que da lugar a la prescripción de antibióticos alternativos, desencadenando otros problemas como la resistencia a los antimicrobianos.

El diagnóstico de este tipo de alergias incluye el uso de pruebas *in vivo* no satisfactorias ya que consumen mucho tiempo y conllevan el riesgo de provocar una nueva reacción alérgica. Por otro lado, los métodos *in vitro* basados en la inmunodetección de IgE alérgeno-específica generan una información muy valiosa, confirmando o descartando la alergia y postulándose como una alternativa de diagnóstico segura. A pesar de ello, las pruebas *in vitro* actuales carecen de sensibilidad y exactitud, con un 81% de falsos negativos, lo que limita su uso diagnóstico.

Esto ha desencadenado una creciente demanda de nuevas pruebas *in vitro* basadas en la inmunodetección de IgE, el biomarcador presente en suero más estudiado en alergia. Sin embargo, la falta de estándares de referencia de IgE alérgeno-específica ha imposibilitado la validación y estandarización de los métodos, lo que hace que los resultados de las distintas pruebas no sean comparables. Además, dicha falta de estándares de referencia hace que las concentraciones de IgE específica se calculen a partir de una curva de calibración de IgE total mediante una interpolación heteróloga, lo que ha demostrado no ser totalmente precisa ya que no se tiene en cuenta la interacción entre el determinante antigénico y la IgE específica.

Por los motivos mencionados, en esta tesis doctoral se plantea como objetivo principal el diseño, obtención y caracterización de proteínas recombinantes con reactividad frente a antibióticos β-lactámicos.

Esta investigación comienza con la puesta a punto de un inmunoensayo en placa ELISA con detección quimioluminiscente para la cuantificación de IgE específica. Se utilizan determinantes antigénicos de penicilina G, penicilina V, amoxicilina y piperacilina conjugados a la proteína Histona H1, consiguiendo finalmente un ensayo multiparamétrico quimioluminiscente. El ensayo desarrollado permitió determinar IgE específica por debajo de 0.1 IU/mL (0.24 ng/mL), identificando pacientes alérgicos con una mayor sensibilidad, utilizando solo 25 µL de muestra (suero). El inmunoensayo se aplicó con éxito en una cohorte de 140 muestras de suero humano, mostrando una buena sensibilidad (64.6%), así como una excelente especificidad clínica (100%), que mejoran significativamente el carácter predictivo de las pruebas *in vitro* existentes.

A continuación, se abordó la obtención y caracterización de proteínas recombinantes similares a las IgE específicas de los β-lactámicos utilizando la metodología *Phage Display*. La primera aproximación se trata de una proteína que mimetiza el comportamiento de una IgE específica. Se trata de una construcción recombinante formada por dos anticuerpos de dominio único (nanoanticuerpos) unidos a través de un enlazador peptídico corto. De esta manera, se obtuvieron proteínas recombinantes en las que uno de los nanoanticuerpos reconoce selectivamente un determinante antigénico de un antibiótico β-lactámico (penicilina G, amoxicilina y aztreonam), mientras que el otro reconoce específicamente al parátopo de un anticuerpo detector anti-IgE (Omalizumab). Estas construcciones resultaron ser estables y funcionales.

El papel de estas proteínas recombinantes como calibrador homólogo se estudió determinando la concentración de IgE específica a penicilina G presente en suero mediante un ensayo quimioluminiscente. Se analizaron 65 muestras procedentes de pacientes alérgicos y controles, lo que permitió comprobar que el método desarrollado duplicaba la sensibilidad clínica (66%) frente al método de referencia (28%), mientras que mantenía una especificidad clínica del 100%. La sensibilidad analítica alcanzada en el ensayo era inferior a 0.1 IU/mL y presentaba una excelente reproducibilidad (RSD < 10%). Esta mejora en la sensibilidad permite abordar de forma más fiable el etiquetado correcto de los alérgicos a estos antibióticos, así como el desetiquetado de aquellos que no lo son.

Alcanzado este punto, la tesis se centró en la producción de IgE específica recombinante utilizando como material de partida ADN de un paciente alérgico a

amoxicilina y penicilina G, en combinación con la metodología *Phage Display* y el sistema de expresión de baculovirus en células de insecto. A partir del material genético aislado, se obtuvo una biblioteca inmune con la que se realizó *Phage Display* y se aislaron los clones que expresaban fragmentos scFv específicos a amoxicilina y penicilina G. Posteriormente, mediante ingeniería de anticuerpos y métodos de expresión en células de insecto se consiguió producir una IgE específica recombinante. De esta manera, se obtuvo un producto biológico que cumple con los requisitos para su adopción como material de referencia en ensayos *in vitro*: estabilidad, especificidad, seguridad biológica y capacidad de producción en masa. Esta proteína recombinante satisface la necesidad que existe actualmente de disponer de patrones o material de referencia específicos con el que validar nuevos ensayos, así como para poder realizar una estandarización de los métodos existentes. Igualmente, se utilizó como calibrador homólogo para la determinación de IgE específica a amoxicilina presente en 150 muestras representativas. Se alcanzó un límite de detección de 0.05 IU/mL con el que se conseguía un aumento de la sensibilidad clínica (73%) que cuadruplicaba al método de referencia (16%), manteniendo la especificidad clínica en el 100%.

De esta forma, se ha conseguido producir y caracterizar proteínas recombinantes a partir de determinantes antigénicos asociados a reacciones alérgicas a antibióticos β-lactámicos y desarrollar estrategias analíticas basadas en fundamentos inmunoquímicos que consiguen mejorar la sensibilidad clínica de los ensayos *in vitro*, convirtiéndolas en herramientas fiables y eficaces a la hora de determinar si un paciente es alérgico a los antibióticos β-lactámicos.

ABSTRACT

β-lactam antibiotics are the most widely used drugs worldwide to treat bacterial infections due to their efficacy, safety, and cost, accounting for 65% of the global antibiotic market. However, these drugs, including penicillin and all its derivatives, are capable of generating an allergic response, being the most common cause of immune-mediated drug reactions. In fact, up to 10% of the general population claims to be allergic to some of these antibiotics. However, a high percentage of patients labeled as allergic would be classified as tolerant after an accurate clinical evaluation. That is why initiatives to de-label misclassified patients are now part of stewardship programs for these drugs. This prevents misclassification that results in the prescription of alternative antibiotics, triggering other problems such as antimicrobial resistance.

The diagnosis of this type of allergy involves using unsatisfactory *in vivo* tests that are time-consuming and carry the risk of triggering a new allergic reaction. On the other hand, *in vitro* methods based on allergen-specific IgE immunodetection generate precious information, confirming or ruling out allergies and postulating themselves as a safe diagnostic alternative. Despite this, current *in vitro* tests lack sensitivity and accuracy, with 81% false negatives limiting their diagnostic use.

This has led to a growing demand for new *in vitro* tests based on the immunodetection of IgE, the most studied biomarker in serum allergy. However, the lack of reference standards for allergen-specific IgE has made it impossible to validate and standardize methods, which means that the results of the different tests are not comparable. Furthermore, this lack of reference standards means that specific IgE concentrations are calculated from a total IgE calibration curve by heterologous interpolation, which has been shown not to be entirely accurate as it does not consider the interaction between the antigenic determinant and specific IgE.

For the reasons mentioned above, the main objective of this doctoral thesis is to design, obtain and characterize recombinant proteins with reactivity against β-lactam antibiotics.

This research begins with developing an ELISA immunoassay with chemiluminescent detection for quantifying specific IgE. Antigenic determinants of penicillin G, penicillin V, amoxicillin and piperacillin conjugated to Histone H1 protein are used, finally achieving a chemiluminescent multiparametric assay. The assay

developed allowed the determination of specific IgE below 0.1 IU/mL (0.24 ng/mL), identifying allergic patients with greater sensitivity, using only 25 μL of sample (serum). The immunoassay was successfully applied on a cohort of 140 human serum samples, showing good sensitivity (64.6%) and excellent clinical specificity (100%), significantly improving the predictive character of existing *in vitro* tests.

Next, the obtaining and characterizing recombinant proteins similar to β-lactam-specific IgE was approached using the *Phage Display* methodology. The first approach deals with a protein that mimics the behavior of a specific IgE. It is a recombinant construct consisting of two single-domain antibodies (nanobodies) linked through a short peptide linker. In this way, recombinant proteins were obtained in which one of the nanobodies selectively recognizes an antigenic determinant of a β-lactam antibiotic (penicillin G, amoxicillin and aztreonam), while the other specifically recognizes the paratope of an anti-IgE detector antibody (Omalizumab). These constructs were found to be stable and functional.

The role of these recombinant proteins as a homologous calibrator was studied by determining the concentration of penicillin G-specific IgE present in serum by employing a chemiluminescent assay. Sixty-five samples from allergic patients and controls were analyzed, which showed that the developed method doubled the clinical sensitivity (66%) compared to the reference method (28%), while maintaining a clinical specificity of 100%. The analytical sensitivity achieved in the assay was less than 0.1 IU/mL and showed excellent reproducibility (RSD < 10%). This improved sensitivity makes it possible to more reliably address the correct labeling of those allergic to these antibiotics and the delabeling of those who are not.

At this point, the thesis focused on the production of recombinant specific IgE using as starting material the DNA of a patient allergic to amoxicillin and penicillin G, in combination with the *Phage Display* methodology and the baculovirus expression system in insect cells. From the isolated genetic material, an immune library was obtained with which Phage Display was performed and clones expressing scFv fragments specific to amoxicillin and penicillin G were isolated. Subsequently, by means of antibody engineering and expression methods in insect cells, a recombinant-specific IgE was produced. In this way, a biological product was obtained that meets the requirements for its adoption as reference material in *in vitro* assays: stability, specificity, biological safety

and mass production capacity. This recombinant protein satisfies the current need for specific standards or reference material with which to validate new assays, as well as to standardize existing methods. Likewise, it was used as a homologous calibrator for the determination of specific IgE to amoxicillin present in 150 representative samples. A detection limit of 0.05 IU/mL was achieved, which increased clinical sensitivity (73%) fourfold compared to the reference method (16%), while maintaining clinical specificity at 100%.

In this way, it has been possible to produce and characterize recombinant proteins from antigenic determinants associated with allergic reactions to β-lactam antibiotics and to develop analytical strategies based on immunochemical fundamentals that improve the clinical sensitivity of *in vitro* assays, making them reliable and effective tools for determining whether a patient is allergic to β-lactam antibiotics.

RESUM

Els antibiòtics β-lactàmics són els fàrmacs més utilitzats en tot el món per a tractar infeccions bacterianes, a causa de la seua eficàcia, seguretat i cost, representen el 65% del mercat mundial d'antibiòtics. Tot i així, aquests fàrmacs, entre els quals es troben la penicil·lina i tots els seus derivats, són capaços de generar una resposta al·lèrgica, sent la causa més comuna de reaccions farmacològiques immunomediades. De fet, fins a un 10% de la població general declara que és al·lèrgica a algun d'aquests antibiòtics. No obstant això, un alt percentatge dels pacients etiquetats com a al·lèrgics es classificarien com a tolerants després d'una avaluació clínica precisa. És per això que eliminar l'etiqueta dels pacients mal catalogats mitjançant les iniciatives de desetiquetatge forma part dels programes d'administració d'aquests fàrmacs, el que evita una classificació errònia que donaria lloc a la prescripció d'antibiòtics alternatius, fet que desencadenaria altres problemes com la resistència als antimicrobians.

El diagnòstic d'aquest tipus d'al·lèrgies inclou l'ús de proves *in vivo* no satisfactòries, ja que consumeixen molt de temps i comporten el risc de provocar una nova reacció al·lèrgica. D'altra banda, els mètodes *in vitro* basats en la immunodetecció d'IgE al·lergen-específica generen una informació molt valuosa, confirmant o descartant l'al·lèrgia i postulant-se com una alternativa de diagnòstic segura. Tanmateix, a les proves *in vitro* actuals hi ha una manca de sensibilitat i exactitud, amb un 81% de falsos negatius, cosa que en limita l'ús diagnòstic.

Tot això ha desembocat en una demanda creixent de noves proves *in vitro* basades en la immunodetecció d'IgE, el biomarcador present en sèrum més estudiat en al·lèrgia. No obstant això, la manca d'estàndards de referència d'IgE al·lergen-específica ha impossibilitat la validació i estandardització dels mètodes, cosa que fa que els resultats de les diferents proves no siguen comparables. A més, aquesta manca d'estàndards de referència fa que les concentracions d'IgE específica es calculen a partir d'una corba de calibratge d'IgE total mitjançant una interpolació heteròloga, un mètode que ha demostrat no ser totalment correcte ja que no es pren en consideració la interacció entre el determinant antigènic i la IgE específica.

Pels motius mencionats, en aquesta tesi doctoral es planteja com a objectiu principal el disseny, l'obtenció i la caracterització de proteïnes recombinants amb reactivitat davant dels antibiòtics β-lactàmics.

Aquesta investigació comença amb la posada a punt d'un immunoassaig en placa ELISA amb detecció quimioluminiscent per a la quantificació d'IgE específica. S'utilitzen determinants antigènics de penicil·lina G, penicil·lina V, amoxicilina i piperacilina conjugats a la proteïna Histona H1, aconseguint finalment un assaig multiparamètric quimioluminiscent. L'assaig desenvolupat va permetre determinar IgE específica per baix de 0.1 IU/mL (0.24 ng/mL), el que permet la identificació de pacients al·lèrgics amb més sensibilitat, utilitzant només 25 μL de mostra (sèrum). L'immunoassaig es va aplicar amb èxit en una cohort de 140 mostres de sèrum humà, mostrant una bona sensibilitat (64.6%), així com una excel·lent especificitat (100%), que milloren significativament el caràcter predictiu de les proves *in vitro* existents.

A continuació, es va abordar l'obtenció i la caracterització de proteïnes recombinants similars a les IgE específiques dels β-lactàmics utilitzant la metodologia *Phage Display*. La primera aproximació és una proteïna que mimetitza el comportament d'una IgE específica. Es tracta d'una construcció recombinant formada per dos anticossos de domini únic (nanoanticossos) units a través d'un enllaçador peptídic curt. D'aquesta manera, es van obtenir proteïnes recombinants en què un dels nanoanticossos reconeix selectivament un determinant antigènic d'un antibiòtic β-lactàmic (penicil·lina G, amoxicilina i aztreonam), mentre que l'altre reconeix específicament el paràtop d'un anticòs detector anti-IgE (Omalizumab). Aquestes construccions van resultar estables i funcionals.

El paper d'aquestes proteïnes recombinants com a calibrador homòleg es va estudiar determinant la concentració d'IgE específica a penicil·lina G present en sèrum mitjançant un assaig quimioluminiscent. Es van analitzar 65 mostres procedents de pacients al·lèrgics i controls, cosa que va permetre comprovar que el mètode desenvolupat duplicava la sensibilitat clínica (66%) respecte del mètode de referència (28%), mentre que mantenia una especificitat clínica del 100%. La sensibilitat analítica assolida a l'assaig era inferior a 0.1 IU/mL i presentava una excel·lent reproductibilitat (RSD < 10%). Aquesta millora en la sensibilitat permet abordar de manera més fiable l'etiquetatge correcte dels al·lèrgics a aquests antibiòtics, així com el desetiquetatge dels que no ho són.

Assolit aquest punt, la tesi es va centrar en la producció d'IgE específica recombinant utilitzant com a material de partida l'ADN d'un pacient al·lèrgic a amoxicil·lina i penicil·lina G, en combinació amb la metodologia *Phage Display* i el sistema d'expressió

de baculovirus en cèl·lules d'insecte. A partir del material genètic aïllat, es va obtenir una biblioteca immune amb la qual es va realitzar *Phage Display* i es van aïllar els clons que expressaven fragments scFv específics a amoxicil·lina i penicil·lina G. Posteriorment, mitjançant enginyeria d'anticossos i mètodes d'expressió en cèl·lules d'insecte, es va aconseguir produir una IgE específica recombinant. D'aquesta manera, es va obtenir un producte biològic que compleix els requisits per ser adoptat com a material de referència en assajos *in vitro*: estabilitat, especificitat, seguretat biològica i capacitat de producció en massa. Aquesta proteïna recombinant satisfà la necessitat que existeix actualment de disposar de patrons o material de referència específics amb els quals validar nous assajos, així com per a poder realitzar una estandardització dels mètodes existents. Igualment, es va utilitzar com a calibrador homòleg per a la determinació d'IgE específica a amoxicil·lina present en 150 mostres representatives. Es va assolir un límit de detecció de 0.05 IU/mL amb què s'aconseguia un augment de la sensibilitat clínica (73%) que quadriplicava al mètode de referència (16%), mantenint l'especificitat clínica al 100%.

Amb el sistema proposat, s'ha aconseguit produir i caracteritzar proteïnes recombinants a partir de determinants antigènics associats a reaccions al·lèrgiques a antibiòtics β-lactàmics i desenvolupar estratègies analítiques basades en fonaments immunoquímics que aconsegueixen millorar la sensibilitat clínica dels assajos *in vitro*, convertint-los en eines fiables i eficaces a l'hora de determinar si un pacient és al·lèrgic als antibiòtics β-lactàmics.

Diseminación de resultados relacionados con esta tesis

Los resultados derivados de esta tesis han dado lugar a las siguientes contribuciones científicas.

ARTÍCULOS PUBLICADOS EN REVISTAS CIENTÍFICAS INDEXADAS:

Autores: P. Quintero-Campos, M.J. Juárez, S. Morais, A. Maquieira,
Título: Multiparametric Highly Sensitive Chemiluminescence Immunoassay for Quantification of β-Lactam-Specific Immunoglobulin E.
Revista: *Analytical Chemistry.* 92, 14608–14615 (2020).

Autores: P. Segovia-de los Santos, **P. Quintero-Campos**, S. Morais, C. Echaides, A. Maquieira, G. Lassabe, G. González-Sapienza.
Título: Bispecific Single-Domain Antibodies as Highly Standardized Synthetic Calibrators for Immunodiagnosis.
Revista: *Analytical Chemistry.* 94, 1342–1349 (2022).

Autores: P. Quintero-Campos, P. Segovia-de los Santos, E. Ibáñez-Echevarria, D. Hernández-Fernández de Rojas, P. Casino, G. Lassabe, G. González-Sapienza, A. Maquieira, S. Morais.
Título: An ultra-sensitive homologous chemiluminescence immunoassay to tackle penicillin allergy.
Revista: *Analytica Chimica Acta.* 1214, 339940 (2022).

COMUNICACIONES EN CONGRESOS NACIONALES E INTERNACIONALES

Título: Inmunoensayo con detección luminiscente para la determinación de IgE específica de alergia a β-lactámicos.
Autores: P. Quintero-Campos, S. Morais, A. Maquieira.
Congreso: XII International Workshop on Sensors and Molecular Recognition
Lugar: València, España.
Fecha: 05/07/2018

Título: Detección *in vitro* de alergia a amoxicilina mediante inmunoensayo luminiscente.
Autores: P. Quintero-Campos, S. Morais, A. Maquieira.
Congreso: XIII International Workshop on Sensors and Molecular Recognition
Lugar: València, España.
Fecha: 04/07/2019

Título: *In vitro* detection of amoxicillin allergy by luminescent immunoassay
Autores: P. Quintero-Campos, M. J. Juárez, S. Morais, A. Maquieira.
Congreso: European Academy of Allergy and Clinical Immunology Congress (EAACI)
Lugar: Londres, Reunio Unido. (ON-LINE)
Fecha: 08/06/2020

Título: Highly improved sensitive *in vitro* drug allergy test using a collection of new beta-lactam protein antigens.
Autores: M.J. Juárez, **P. Quintero-Campos**, S. Morais, A. Maquieira.
Congreso: European Academy of Allergy and Clinical Immunology Congress (EAACI)
Lugar: Londres, Reunio Unido. (ON-LINE)
Fecha: 08/06/2020

Título: Moving towards advanced in *in vitro* diagnostics for drug allergy
Autores: S. Morais, M. J. Juárez, S.r Mas, **P. Quintero-Campos**, E. Peña, M. E. Fernández, L. A. Tortajada, A. Maquieira.
Congreso: Rapid Methods Europe 2021
Lugar: ON-LINE
Fecha: 03/02/2021

Título: Desarrollo de nanoanticuerpos para el diagnóstico *in vitro* de alergia
Autores: P. Quintero-Campos, S. Morais, A. Maquieira.
Congreso: XIV International Workshop on Sensors and Molecular Recognition
Lugar: València, España.
Fecha: 08/07/2021

Título: Towards the standardisation of in vitro diagnostic testing for drug allergy
Autores: S. Morais, **P. Quintero-Campos**, M.J. Juárez, A. Maquieira.
Congreso: XXXVIII Reunión Bienal de la Real Sociedad Española de Química.
Lugar: Granada, España.
Fecha: 29/06/2022

Título: Producción de suero artificial con reactividad a antibióticos β-lactámicos mediante tecnologías recombinantes
Autores: P. Quintero-Campos, R. Gozalbo-Rovira, J. Rodríguez-Díaz, S. Morais, A. Maquieira.
Congreso: XV International Workshop on Sensors and Molecular Recognition.
Lugar: València, España.
Fecha: 11/07/2022
Reconocimiento: 1er premio póster

Título: Production of artificial serum with reactivity to amoxicillin.
Autores: P. Quintero-Campos, R. Gozalbo-Rovira, J. Rodríguez-Díaz, S. Morais, A. Maquieira.
Congreso: 14th Rapid Methods Europe Conference.
Lugar: Ámsterdam, Países Bajos.
Fecha: 3/10/2022

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LISTA DE ABREVIATURAS

ADN/DNA	Ácido desoxirribonucleico
ARN/RNA	Ácido ribonucleico
AMX	Amoxicilina
ARNm	Ácido ribonucleico mensajero
AU	*Arbitrary units*: unidades arbitrarias
AZ	Aztreonam
BLC	β-lactámico
BSA	*Bovine serum albumin*: albúmina sérica bovina
CD	*Cluster of differentiation*: grupo de diferenciación
CDR	*Complementarity-determining region*: región determinante de la complementariedad
CFSE	Éster de succinimidil-carboxifluoresceína
CLIA	*Chemiluminescence immunoassay*: inmunoensayo quimioluminiscente
CLV	Ácido clavulánico
DDD	*Defined daily doses*: dosis diaria definida
ELISA	*Enzyme-linked immunosorbent assay*: ensayo por inmunoadsorción ligado a enzimas
Fc	Fracción cristalizable de las inmunoglobulinas
FcRI	Receptor de alta afinidad de las inmunoglobulinas
FEIA	*Fluorimetric enzyme-linked immunoassay*: inmunoensayo fluorimétrico ligado a enzimas
H1	Histona H1
HcAbs	*Heavy chain-only antibodies*: anticuerpos de cadena pesada
HIS	Histidina
hmCLIA	*Homologous chemiluminescence immunoassay*: imunoensayo quimioluminiscente homólogo
HRP	*Horseradish peroxidase*: peroxidasa del rábano picante
HSA	*Human serum albumin*: albúmina sérica humana
ICAP	ImmunoCAP
IgE	Inmunoglobulina E
IgG	Inmunoglobulina G
IgM	Inmunoglobulina M
IL	Interleucina
IPTG	Isopropil-β-D-1-tiogalactopiranósido
IU	*International units*: unidades internacionales

KLH	*Keyhole limpet hemocyanin: h*emocianina de *Megathura crenulata*
LOD	*Limit of Detection*: límite de detección
LOQ	*Limit of Quantification*: límite de cuantificación
mAb	*Monoclonal antibody*: anticuerpo monoclonal
MHC	*Major histocompatibility complex*: complejo mayor de histocompatibilidad
Nb	*Nanobody*: nanoanticuerpo
NIR	*Non immediate reaction*: reacciones no inmediatas
OD	Densidad óptica
Om	Omalizumab
OMS/WHO	Organización Mundial de la Salud.
PBP	*Penicillin-binding proteins*: proteínas de unión a la penicilina
PCR	*Polymerase chain reaction*: reacción en cadena de la polimerasa
PEG	Polietilenglicol
PG	Penicilina G
POH	*Perioperative hypersensitivity*: hipersensibilidad en el perioperatorio
PPD	Prueba de provocación directa
PRAN	Plan nacional frente a la resistencia a antibióticos
PROA	Programa de optimización de uso de antibióticos
PSU	*Penicillin standardised unit*: unidad estandarizada de penicilina
RAM	Reacciones adversas a medicamentos
RAST	*Radio allergo sorbent test*: prueba de radioalergoabsorbancia
RHF	Reacciones de hipersensibilidad a fármacos
RIA	Radioinmunoensayo
rsIgE	*Recombinant specific IgE*: IgE específica recombinante
SDS-PAGE	*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis*: electroforesis en gel de poliacrilamida con dodecilsulfato sódico
sIgE	*Specific immunoglobulin E*: imunoglobulina E específica
ST	*Skin Test*: prueba cutánea
TMB	3, 3', 5, 5'-tetrametilbenzidina
VH	Dominio de cadena pesada
VHH	Dominio único de cadena pesada
VPP	Valor predictivo positivo

1. INTRODUCCIÓN

1.1. ALERGIA A ANTIBIÓTICOS β-LACTÁMICOS

1.1.1. Antibióticos β-lactámicos.

Uno de los grandes hitos del siglo XX fue el descubrimiento de la penicilina por parte de Alexander Fleming en 1928 cuando observó que el hongo *Penicillium notatum* producía un compuesto bactericida que provocaba lisis y reducía la tasa de crecimiento de *Staphylococcus aureus*. Tras su identificación, en 1940 Ernst Boris Chain y Lord Howard Florey retomaron el trabajo de Fleming, consiguiendo aislar la penicilina y escalar su producción a nivel industrial, lo que se tradujo en la utilización del antimicrobiano a nivel mundial, y lo que les valió el Premio Nobel en Fisiología o Medicina en 1945.[1]

Estos grandes avances dieron lugar al desarrollo de la familia de los antibióticos β-lactámicos (BLC). Esta familia de antibióticos está compuesta por la penicilina y todos sus derivados naturales y sintéticos, y ha ido ampliándose con la finalidad de aumentar su espectro de acción. Los antibióticos BLCs se clasifican en 5 grupos y todos ellos se caracterizan por poseer un anillo de cuatro miembros (anillo β-lactámico), que les proporciona actividad antibacteriana al inhibir la síntesis de la pared celular. En las penicilinas el anillo BLC se condensa con un anillo de tiazolidina, en las cefalosporinas con un anillo de dihidrotiazina, en los carbapenems con un anillo de dihidropirrol, y en las clavamas con un anillo de oxazolidina. Los dos anillos fusionados en conjunto (la estructura bicíclica) constituyen el núcleo, o región nuclear, de cada grupo de antibiótico BLC.[2] Además, todos estos antibióticos presentan diferentes sustituyentes R, también llamados cadenas laterales. En todos los casos, una cadena lateral (R o R_1) está unida al anillo BLC, y en las cefalosporinas y los carbapenems aparecen cadenas laterales adicionales (R_2 y/o R_3) asociadas al segundo anillo.[3]

Las diferencias en la estructura química de la región nuclear y la cadena lateral han permitido disponer de una gran gama de antibióticos BLCs (Figura 1) con amplio espectro de acción.[2] Esta propiedad ha convertido a estos fármacos en la principal elección para el tratamiento de un gran número de infecciones bacterianas[4], por lo que son antimicrobianos clave desde el punto de vista clínico, tanto en medicina humana como veterinaria.[5,6] Se calcula que el gasto anual en estos antibióticos supera los 15.000 millones de euros y constituye a nivel mundial el 65% del mercado total de antibióticos.[7]

Sin embargo, su uso está intrínsecamente asociado a problemas de gran envergadura. Por un lado, la excesiva utilización de estos fármacos en ganadería y acuicultura

contribuye a una liberación al medio ambiente, especialmente en agua, donde se encuentran residuos de estos, potenciando la aparición de resistencia a los antibióticos al dar lugar a bacterias superresistentes, lo que supone un grave riesgo para la salud de los seres humanos y animales.[8] Pero además, en algunos casos, la ingesta de estos medicamentos puede desencadenar una serie de alteraciones en el organismo que en condiciones normales no provocarían ninguna afección. Es decir, el agente terapéutico se convierte en el problema al originar una cadena de reacciones inesperadas y no deseadas.

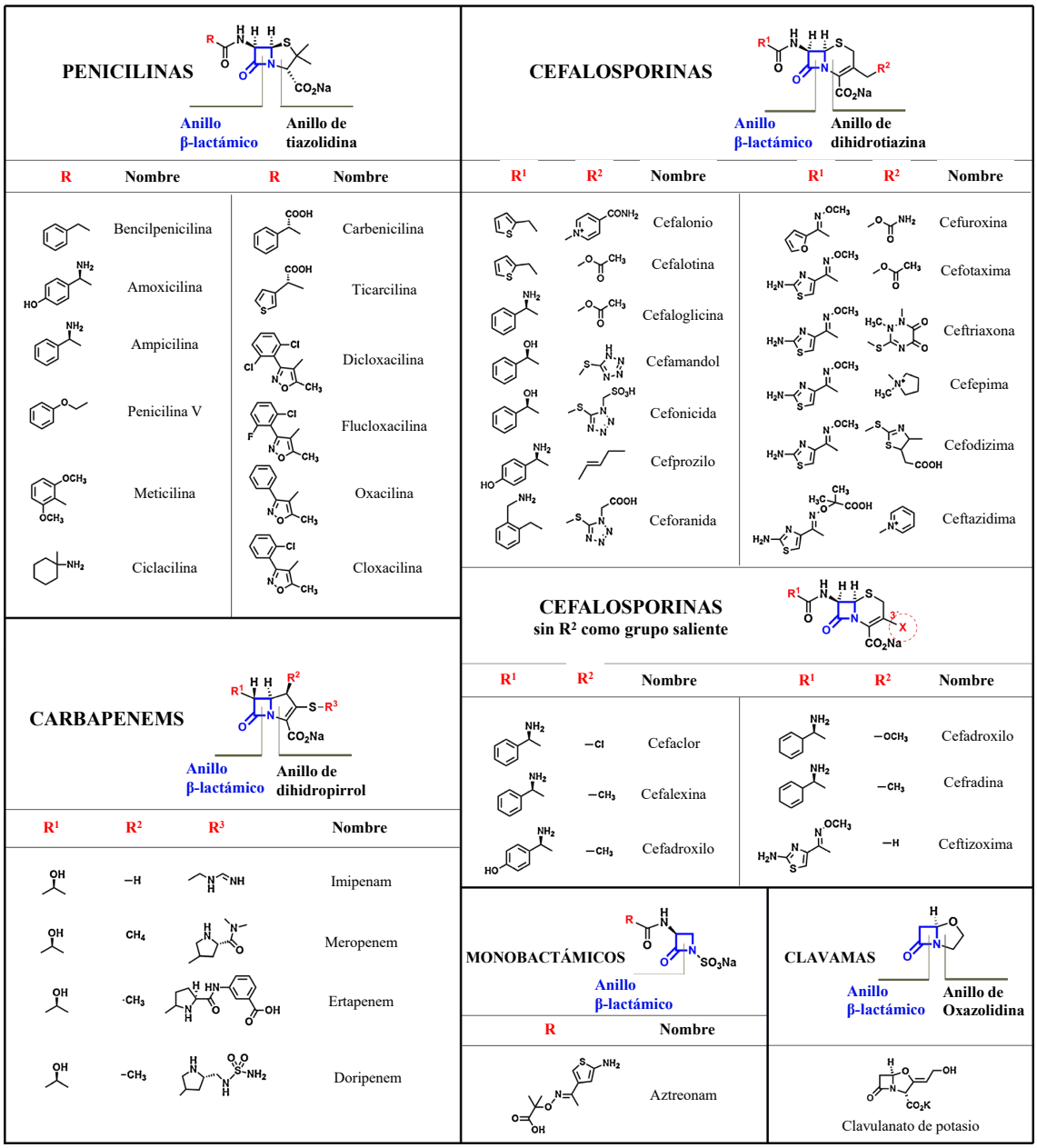

Figura 1: Principales familias de antibióticos β-lactámicos.[3]

1.1.1.1. Mecanismo de acción

Las propiedades antimicrobianas de los antibióticos BLCs reside en la capacidad de inhibir la actividad catalítica de las enzimas transpeptidasas que intervienen en la biosíntesis del peptidoglicano que forma la pared celular bacteriana.[1] Por ello, estas enzimas también se denominan "proteínas de unión a la penicilina" (PBP). La actividad inhibidora de los antibióticos BLCs se basa en las similitudes estructurales y estereoquímicas (Figura 2) de los enlaces amida de la penicilina y el sustrato de la enzima (dipéptido D-Ala-D-Ala) necesario en la primera etapa de la síntesis del peptidoglicano.[9]

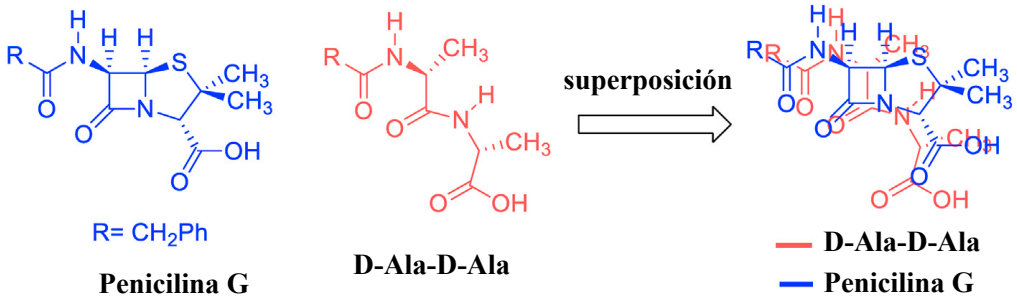

Figura 2: Semejanza estructural entre la penicilina y el sustrato de la PBP.[1]

El reconocimiento de los antibióticos BLCs por la PBP es quimioselectivo. Esta enzima en presencia de este grupo de antibióticos forma un complejo penicilloil-enzima (Figura 3), que por razones estéricas imposibilita la reacción de desacetilación catalizada por la transpeptidasa.[10] La inhibición de la enzima es irreversible, ya que se produce un ataque nucleofílico del residuo de serina presente en el sitio activo de la enzima al grupo carbonilo del anillo BLC. El carbonilo del anillo es altamente reactivo presentando un mayor carácter electrofílico que el carbonilo de la amida presente en la cadena lateral de la estructura general de la penicilina y otros antibióticos BLCs. En consecuencia, el anillo BLC es el principal farmacóforo de este grupo de antibióticos.[11]

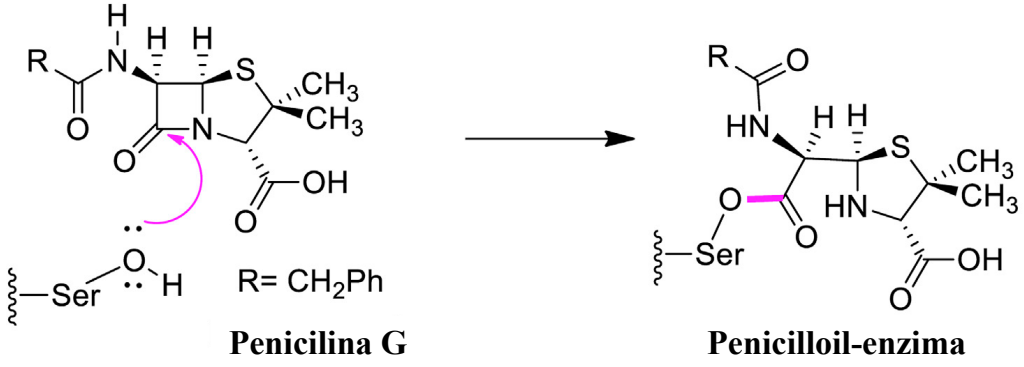

Figura 3: Formación del complejo penicilloil-enzima.[1]

La inhibición de la PBP hace que los enlaces cruzados que se forman en el peptidoglicano sean débiles, lo que provoca que las bacterias sean altamente susceptibles a lisis y muerte celular, lo que resulta en una disminución del crecimiento bacteriano.[1] Esto confiere a los antibióticos BLCs su clasificación como agentes bactericidas.

1.1.2. Hipersensibilidad a los β-lactámicos

Las reacciones adversas a medicamentos (RAM) se definen como cualquier reacción nociva no intencionada que aparece al administrar dosis normales usadas en humanos para la profilaxis, diagnóstico, tratamiento, o para modificar las funciones fisiológicas".[12] Este tipo de reacciones poseen una incidencia del 10%[13] y tienen asociadas morbilidad y mortalidad, lo que las hace peligrosas y requieren de tratamientos económicamente costosos.[14]

Según la clasificación de Rawlins y Thompson[15], las reacciones de hipersensibilidad a Fármacos (RHF) son reacciones adversas de tipo B y, aunque solo constituyen el 15% de las RAM, son graves.[16] Según la clasificación de Gell y Coombs, las RHF se pueden subclasificar en 4 categorías. Las de tipo I (o reacciones de hipersensibilidad) que están mediadas por IgE específica (sIgE); las de tipo II mediadas por mecanismos citotóxicos; las de tipo III conducidas por inmunocomplejos; y las de tipo IV que están mediadas por linfocitos T.[17,18]

La alergia a los antibióticos BLCs se clasifica como RHF de tipo I, es decir, se trata de una reacción de hipersensibilidad inmediata que está mediada por IgE específica al antígeno/fármaco.[19] Sin embargo, los antibióticos BLCs son moléculas pequeñas (<1000 Da) que, por sí mismas, son poco reconocidas por el sistema inmunitario. A pesar de ello, pueden volverse inmunogénicos cuando su forma nativa o sus metabolitos se unen a proteínas séricas o tisulares formando conjugados o aductos hapteno-proteína. En el caso de los antibióticos BLCs, bajo condiciones fisiológicas, tiene lugar la apertura del anillo BLC originando los determinantes -lloyl. El aducto fármaco-proteína se forma debido al ataque nucleofílico en el carbonilo del anillo por parte de los grupos amino de las proteínas (Figura 4).[16] Los determinantes -lloyl son considerados los determinantes mayoritarios al ser responsables de casi el 95% de reacciones alérgicas a antibióticos BLCs.[20] Sin embargo, también existen otros determinantes, clasificados como minoritarios, que son igual de importantes ya que están implicados en reacciones alérgicas graves.[20]

La producción de anticuerpos monoclonales ha ayudado a caracterizar los determinantes antigénicos identificándose tres epítopos diferentes: la cadena lateral, el anillo BLC y la parte que resulta de la conjugación del anillo con la proteína transportadora.[21]

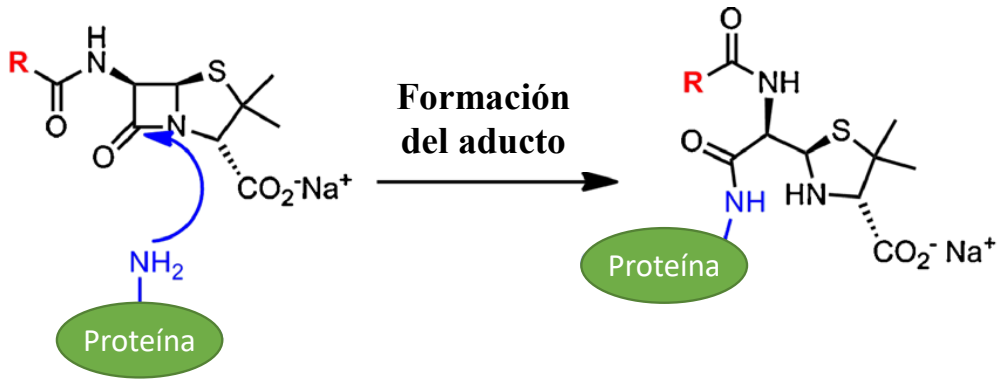

Figura 4: Esquema de la formación aducto fármaco – proteína.[3]

Como se ha mencionado anteriormente, en el caso de la alergia a antibióticos BLCs, la reacción de hipersensibilidad es de tipo I, y esta se divide en dos fases: sensibilización y desencadenamiento (Figura 5).

La fase de sensibilización tiene lugar durante la primera exposición al fármaco. En ella las células dendríticas internalizan el antígeno para presentarlo a los linfocitos T naive (T_H0). En presencia de la IL-4, el T_H0 se convierte en una célula T_H2 específica del fármaco y activa a las células B en presencia de IL-4/IL-13 y CD40/CD40L, haciendo que maduren en células secretoras de IgE específica. Los anticuerpos generados se unen a los FceRIa de los mastocitos de la mucosa y del tejido conectivo, y a los basófilos circulantes. Esta fase suele requerir varios días y los pacientes no desarrollan reacciones durante la primera exposición.[22]

Tras la reexposición al fármaco tiene lugar la fase de desencadenamiento. En ella el entrecruzamiento de los receptores FceRI con múltiples moléculas de IgE provoca la activación de múltiples eventos de señalización química, lo que desencadena la liberación por parte de los mastocitos de mediadores preformados (histamina, heparina, serotonina y mastoproteasas) al espacio extracelular.[23] Las vías de señalización paralelas activadas por la agregación de FceRI convergen en la liberación de las reservas de ácido araquidónico de los mastocitos para promover la síntesis de mediadores eicosanoides, incluyendo prostaglandinas y leucotrienos, que son importantes precursores de la quimiotaxis, la vasodilatación y la broncoconstricción[24].

Aunque la IgE se produce en pequeñas cantidades, la mayor parte de esta se encuentra en los mastocitos tisulares de diferentes órganos, incluidas las paredes de los vasos sanguíneos, lo que explica la naturaleza sistémica de las reacciones alérgicas. Así pues, pequeñas cantidades del fármaco pueden inducir reacciones sistémicas graves, incluida la anafilaxia, dependiendo de la sensibilización del paciente y de la cantidad de IgE específica dirigida contra el fármaco.[25]

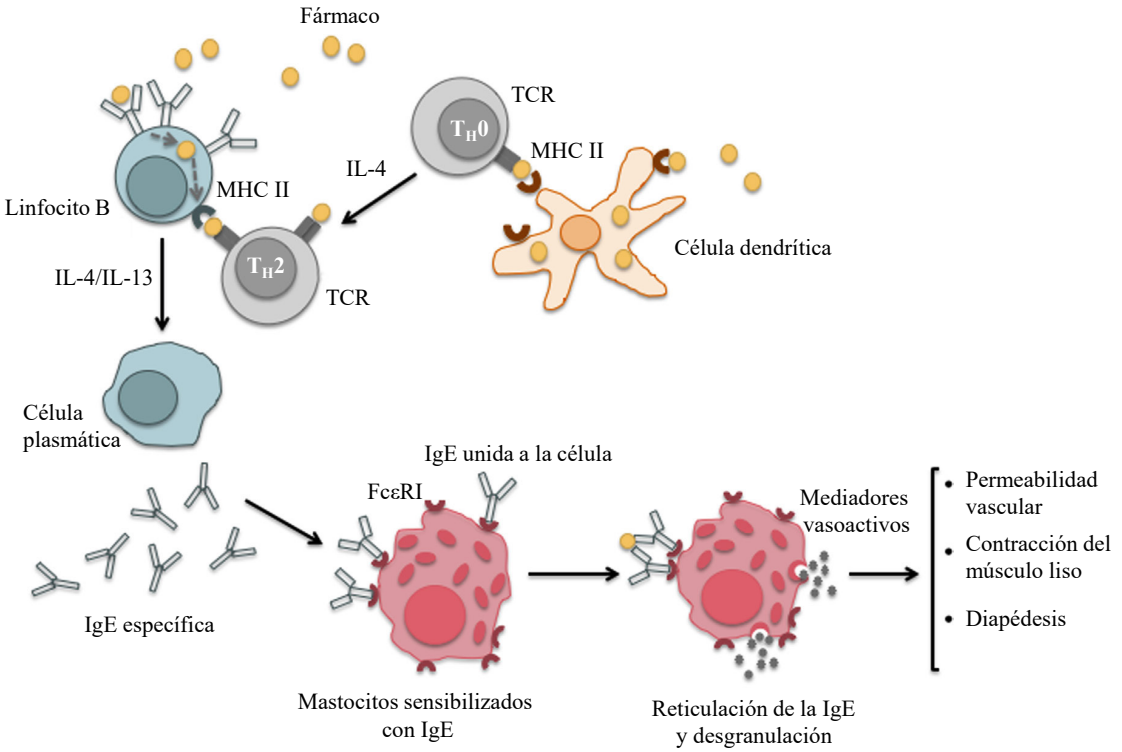

Figura 5: Diagrama de las reacciones de hipersensibilidad de tipo I.[26]

La desgranulación celular y la liberación de mediadores inflamatorios son responsables de los síntomas clínicos. Estos síntomas aparecen entre 1 y 6 horas tras la última administración del fármaco (es decir, son reacciones inmediatas) y suelen manifestarse de forma cutánea (p. ej., picazón, urticaria, angioedema, eritema generalizado), respiratoria (p. ej., congestión nasal, rinorrea, estornudos, ronquera, tos, sibilancias), gastrointestinal (p. ej., náuseas, diarrea, dolor abdominal leve, vómitos) y cardiovascular (p. ej., taquicardia, hipotensión), que pueden aparecer aislados o en combinación como en la anafilaxia.[27]

Los mastocitos y los basófilos pueden ser activados por IgG, sin embargo, se requiere mayor concentración del antígeno para que pueda ser llevada a cabo. Esta diferencia, juega un papel importante como mediador y bloqueante de la anafilaxis mediada por IgE

ya que esta se ve bloqueada cuando la concentración de IgE es baja frente a la concentración de IgG.[28]

Por otro lado, los antibióticos BLCs también pueden dar lugar a reacciones adversas mediadas por células T, que se producen comúnmente después de muchos días de tratamiento, es decir, reacciones no inmediatas (NIR).[29] El exantema maculopapular y la urticaria de aparición retardada son las NIR más frecuentes. Las reacciones adversas cutáneas graves, es decir, la pustulosis exantemática aguda generalizada, el síndrome de Stevens-Johnson, la necrólisis epidérmica tóxica y el síndrome de hipersensibilidad inducida por medicamentos o la erupción por medicamentos con eosinofilia y síntomas sistémicos, son las expresiones más graves de las reacciones no inmediatas a los antibióticos BLCs. [27]

1.1.2.1. Epidemiología

Los estudios epidemiológicos han demostrado que alrededor del 10-25% de la población está etiquetada como alérgica a algún antibiótico BLC.[30,31] Sin embargo, solo un 10% de los pacientes presentan verdadera alergia.[32]

Entre esta familia de antibióticos, la penicilina es la causante de alergia farmacológica más común identificada en los registros médicos, con una prevalencia que oscila entre el 6 y el 25% según la población de tratamiento.[33,34] La aparición de síntomas se asocia a reacciones de hipersensibilidad probablemente relacionadas con el fármaco[35], pero también con interacciones farmacológicas relacionadas con otras infecciones, como cuando un paciente desarrolla una erupción maculopapular a causa de una infección por el virus de Epstein-Barr tras haber sido medicado con una aminopenicilina.[36] De manera que, aproximadamente entre el 0.5% y el 2.0% de las administraciones de antibióticos BLCs dan lugar a una reacción de hipersensibilidad, pero que también podría ser no alérgica.[37] En la actualidad, la tasa de alergia a penicilina mediada por IgE está disminuyendo, lo que podría deberse a la reducción de la administración parental de penicilinas.

En las historias clínicas de los pacientes etiquetados como alérgicos a algún antibiótico BLC el tipo de reacción documentada es "Desconocido" en el 26% de los casos, con otras reacciones comúnmente documentadas, como erupción cutánea (38%), urticaria (18%), angioedema (9%), malestar gastrointestinal (6%), anafilaxia (5%) y prurito (5%).[38] Los pacientes que se presentan para la evaluación de la alergia a estos

antibióticos tienen reacciones informadas similares, siendo la urticaria y la erupción las más comunes.[39] La penicilina ha sido la causa más común de anafilaxia mortal y no mortal inducida por medicamento en los Estados Unidos y el Reino Unido. La tasa más baja de anafilaxia se corresponde a las penicilinas orales, habiéndose registrado un solo caso de anafilaxia mortal por amoxicilina oral en 35 años y 100 millones de tratamientos. [22]

Entre los principales factores de riesgo para alergia a antibiótico BLC se han identificado la administración parental, la exposición frecuente y la edad.[39] Más del 95% de los pacientes que no tienen antecedentes de reacciones alérgicas graves a antibióticos BLCs son tolerantes a los mismos ya que[40] (1) la reacción de hipersensibilidad más comúnmente notificada es una erupción cutánea benigna retardada, probablemente una reacción de hipersensibilidad de tipo IV que puede o no reaparecer cuando los pacientes se vuelven a exponer al antibiótico; (2) la alergia mediada por IgE disminuye con el tiempo, y el 80% de los pacientes se vuelven tolerantes después de una década[41]; y (3) muchos pacientes nunca fueron alérgicos, pero pueden haber tenido una intolerancia u otra causa para los síntomas que pensaban que representaban una reacción al antibiótico BLC, como una infección viral concomitante.[42]

1.1.2.2. Desetiquetado de alergia a β-lactámicos

En el caso de los pacientes etiquetados como alérgicos a algún antibiótico BLC, los facultativos se ven obligados a prescribir antibióticos alternativos a la hora de tratar infecciones bacterianas. Sin embargo, el uso de nuevos antibióticos va intrínsecamente asociado a la posible aparición de nuevas resistencias antimicrobianas. Este problema es considerado actualmente como uno de los grandes retos a los que se enfrentará la sociedad en las próximas décadas. Es para ello que, de forma preventiva, el Ministerio de Sanidad elabora programas de optimización de uso de antibióticos (PROA) dentro del Plan nacional frente a la resistencia a los antibióticos (PRAN). En estas estrategias se prioriza la prescripción selectiva de ciertos antibióticos, entre ellos los BLCs, a la hora de tratar infecciones bacterianas.[43,44] Es por ello por lo que se hace necesario realizar un diagnóstico completo que confirme la alergia. Sin embargo, cuando un paciente presenta algún tipo de reacción inesperada frente a la ingesta del fármaco, inmediatamente desde la atención primaria se le etiqueta como alérgico sin identificar si el antibiótico es la causa o no. De hecho, estudios recientes ponen de manifiesto que solo el 10% de los pacientes etiquetados como alérgicos a algún antibiótico BLC realmente lo son.[22]

Este etiquetado incorrecto se asocia, no solo con un aumento de la resistencia a los antimicrobianos, sino también con un incremento de las infecciones por *Clostridium difficile*, una prolongación de las estancias hospitalarias, un mayor número de ingresos en cuidados intensivos y de reingresos hospitalarios, así como una mayor mortalidad.[33] Más allá de comprometer la salud, etiquetar de forma errónea a los individuos que requieren estos fármacos como alérgicos a un antibiótico BLC, o en ocasiones a más de uno, conlleva unos riesgos sanitarios importantes, además de unos costes significativamente mayores, estimados en más de 60 millones de euros.[34]

Por todo esto, los programas de desetiquetado de la población clasificada como alérgica a antibióticos, especialmente a BLCs, se han convertido en una práctica asistencial a la hora de administrar estos fármacos. En este contexto, una de las principales claves es el método de diagnóstico elegido[45], lo que pone de manifiesto la importancia de desarrollar nuevas pruebas que permitan una clasificación de pacientes más selectiva y fiable.[46]

1.2. MÉTODOS DE DIAGNÓSTICO DE ALERGÍAS A β-LACTÁMICOS

La Academia Europea de Alergia e Inmunología Clínica (EAACI) ha establecido un protocolo diagnóstico que aplicar ante un paciente con sospecha de alergia (Figura 6). Este comienza por el estudio de la historia clínica completa y continúa con diferentes pruebas diagnósticas, normalmente primero con pruebas *in vivo*, seguidas de pruebas *in vitro*.[47]

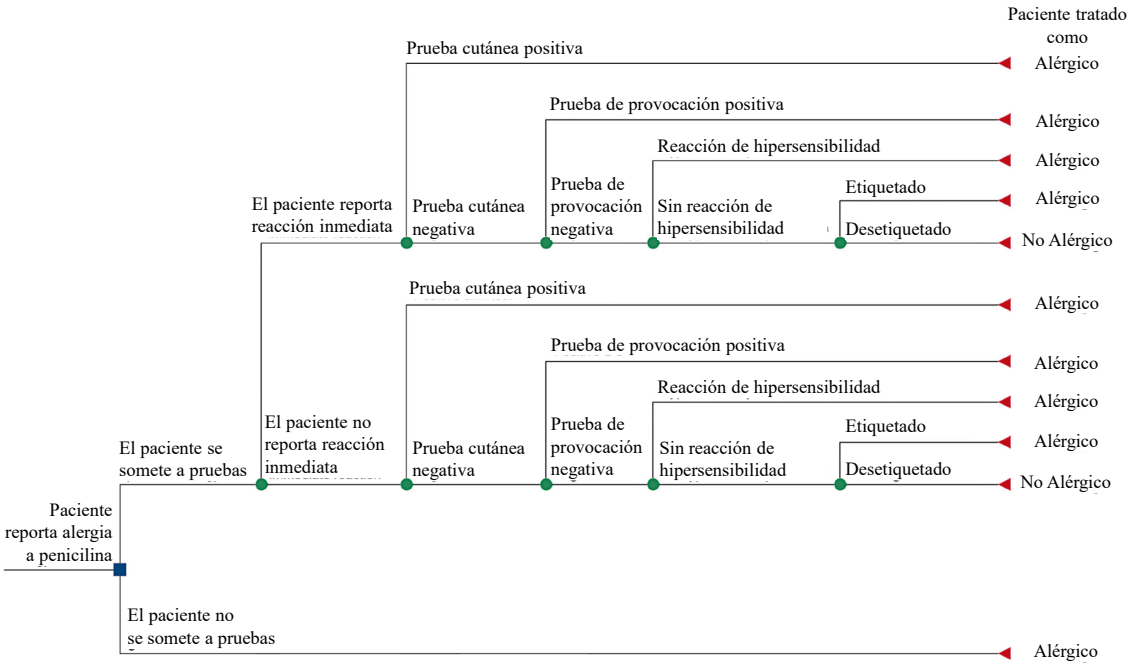

Figura 6: Ejemplo de árbol de decisión para el diagnóstico de alergias a BLCs.[5]

1.2.1. Evaluación del historial clínico

Una historia clínica completa y detallada es el primer paso hacia un diagnóstico preciso de las RHF y evitar así un etiquetado erróneo. Para ello, hay que tener en cuenta el momento de la reacción (con respeto a la administración del fármaco), la naturaleza de los fármacos implicados, los antecedentes de una exposición previa al mismo fármaco o a fármacos con una estructura química similar, los antecedentes médicos/genéticos y las circunstancias de la aparición de la reacción con el fin de realizar un diagnóstico diferencial.[48]

Un análisis detallado de la historia clínica permite diferenciar si los síntomas pueden haber sido simplemente una consecuencia de una enfermedad subyacente, como una infección vírica, o de una interacción entre un antibiótico y un agente infeccioso.[33] Un ejemplo bien caracterizado de esto es el caso de pacientes activamente infectados por el virus de Epstein-Barr que son tratados con ampicilina y desarrollan una erupción

morbiliforme.[36] Sin embargo, a pesar de tener una gran importancia en la valoración del paciente, el historial clínico no es suficiente ni concluyente para establecer un diagnóstico clínico definitivo.[49]

1.2.2. Métodos *in vivo*

Tras el análisis del historial clínico, el siguiente paso para la confirmación o exclusión de la alergia se basa en los resultados de las pruebas *in vivo.* Desde su primer uso en 1921[50], el contacto con un alérgeno y la posterior aparición de picor local, hinchazón y eritema ha sido probablemente el procedimiento diagnóstico más rápido para la evaluación de la sensibilización alérgica.[51]

1.2.2.1. Prueba cutánea

Las pruebas cutáneas (ST, del inglés "Skin Test") (Figura 7) se consideran el mejor método *in vivo* para detectar reacciones a fármacos mediadas por IgE y su combinación con una prueba de provocación se considera el *gold standard* para el diagnóstico de la alergia inmediata.[52] Es, por lo tanto, la primera prueba que se realiza para evaluar las reacciones de hipersensibilidad inmediata. Esta consiste en depositar una gota del alérgeno y pinchar la piel con una lanceta, de manera que la sustancia alergénica penetre en la capa superficial de la piel. La prueba sigue procedimientos operativos estándar y es realizada por personal formado en su práctica e interpretación. Si el resultado de la prueba es positivo a concentraciones validadas y no irritantes, confirman el diagnóstico de sensibilización al fármaco culpable y/o a los fármacos con reactividad cruzada y evitan la necesidad de realizar una prueba de provocación de fármacos.[48]

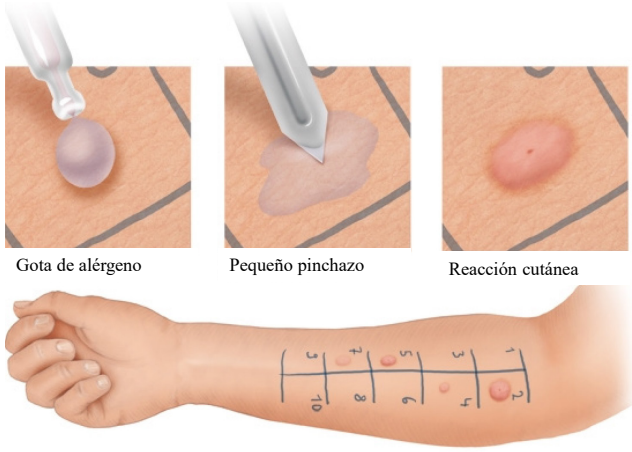

Figura 7: Representación de las etapas de la prueba cutánea.[53]

El panel de reactivos para evaluar las reacciones de hipersensibilidad a antibióticos BLCs por ST se basan en la inmunogenicidad e incluyen determinantes mayoritarios y minoritarios. La penicilloil-polilisina es el determinante mayoritario, mientras que el penilloato, el penicilloato, la penicilina G, la amoxicilina y la ampicilina se agrupan como determinantes menores[33]. Sin embargo, su valor predictivo positivo (VPP) no está claro ya que datos retrospectivos limitados demuestran que el VPP de las pruebas cutáneas es inferior al 50%.[54]

A pesar de su uso habitual en la práctica asistencial y su amplia aceptación, las ST tienen bastante variabilidad[55] y su valor diagnóstico está limitado. En el caso de la realización de ST para el diagnóstico de reacciones inmediatas, estas deben llevarse a cabo tras un intervalo de tiempo de 3 a 6 semanas desde la reacción. Sin embargo, en el caso de los antibióticos BLCs la reactividad cutánea disminuye con el tiempo; por lo que se recomienda realizar la prueba lo antes posible. No obstante, no existe un límite superior de tiempo a partir del que la ST pueda considerarse inexacta.[54]

1.2.2.2. Prueba de provocación directa

Las pruebas de provocación directa (PPD) consisten en la administración controlada de dosis crecientes del fármaco a pacientes con un historial clínico sugestivo de alergia. Se administra el presunto fármaco responsable o un fármaco alternativo relacionado estructural o farmacológicamente. La PPD se suele realizar en un procedimiento a ciegas en pacientes con pruebas de ST e *in vitro* negativas en los que la confirmación del diagnóstico es esencial. Se administran dosis crecientes del fármaco a intervalos de 30-90 minutos hasta completar la dosis terapéutica. Si aparecen síntomas de una reacción alérgica durante la prueba, el procedimiento debe interrumpirse y el paciente es etiquetado como alérgico.[56]

La Red Europea de Alergia a Medicamentos recomienda el uso de las PPD para confirmar reacciones de hipersensibilidad a medicamentos. Dado que la sensibilidad de las ST no es óptima, la PPD debe tenerse en cuenta para establecer el diagnóstico en un porcentaje no despreciable de casos, sin embargo, este procedimiento no se recomienda en pacientes con antecedentes de episodios alérgicos graves.[57] En el caso de niños, para quienes la sensibilidad del ST puede ser inferior al 10%, se recomienda considerar este procedimiento.[58]

1.2.3. Métodos *in vitro*

Aunque son menos sensibles que las pruebas *in vivo*, las pruebas *in vitro* han demostrado ser útiles y complementarias a las pruebas ST y PPD, permitiendo identificar o confirmar el fármaco responsable de la alergia.[59] Además, constituyen la única alternativa a las pruebas *in vivo* y se recomienda que se lleven a cabo en pacientes de alto riesgo, como los pacientes con un historial de reacciones graves.[60] Estas pruebas se basan en la detección de marcadores biológicos asociados a dicha patología.

Su evolución durante los últimos años ha hecho que este tipo de pruebas ofrezcan numerosas ventajas en el diagnóstico de alergia a medicamentos como seguridad, condiciones controladas y, en el caso de las pruebas serológicas, almacenamiento a largo plazo de las muestras.[56] Sin embargo, su uso no está muy extendido debido a algunas desventajas como su menor sensibilidad (y a veces especificidad) en comparación con la ST y el requerimiento de equipos caros y específicos que solo están disponibles en laboratorios centralizados en grandes hospitales con servicios de alergia.[56]

1.2.3.1. ELISpot

El ELISpot (Figura 8) se utiliza en el diagnóstico de NIR y es una técnica basada en la inmunoadsorción ligada a enzima que permite visualizar los productos que se secretan tras la activación celular por el fármaco responsable de la alergia, como citoquinas o marcadores citotóxicos. Cada mancha (spot) que se desarrolla en el ensayo representa una sola célula activa[61]. Este ensayo proporciona información tanto cualitativa (con respecto a la citoquina específica u otra molécula inmune secretada) como cuantitativa (la frecuencia de células que responden dentro de la población de la prueba). Esta técnica presenta dos grandes ventajas: alta sensibilidad[62] y su capacidad de detectar células T reactivas a fármacos incluso varios años después de que se produjese la reacción[63].

El Elispot para el interferón gamma (IFNγ) se ha utilizado para la evaluación de las NIR inducidas por antibióticos BLCs, principalmente para amoxicilina, con una sensibilidad que oscila entre el 13% y el 91%.[62]

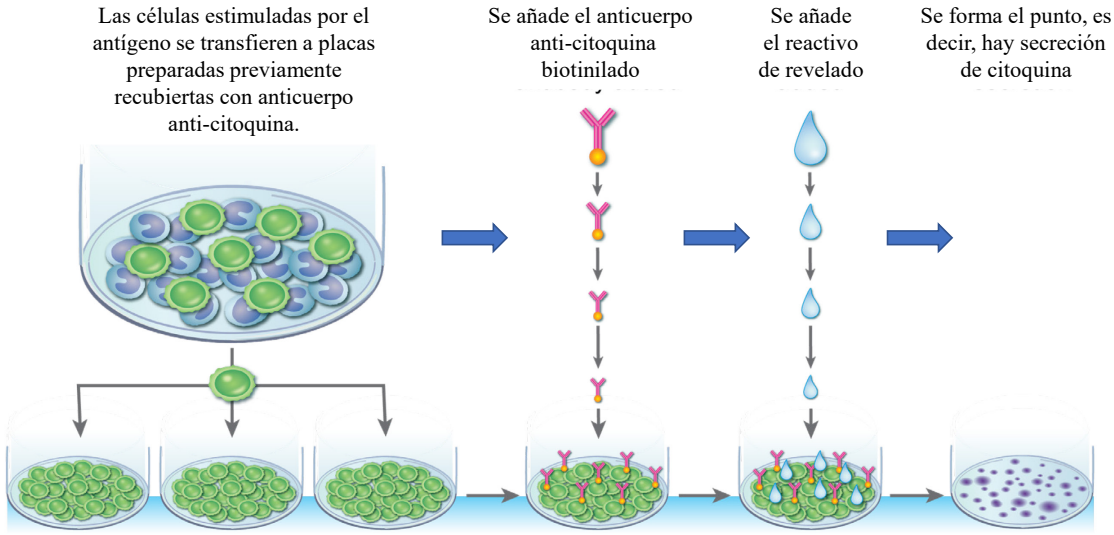

Figura 8: Esquema del ensayo ELISpot.[61]

1.2.3.2. Prueba de transformación de linfocitos

La prueba de transformación de linfocitos (Figura 9) se utiliza en el diagnóstico de NIR y se basa en la proliferación de linfocitos T tras la estimulación con el fármaco.[61] Esta proliferación se mide marcando los linfocitos con un compuesto fluorescente (éster de succinimidil-carboxifluoresceína, CFSE) que se acumula en su citoplasma. Tras el contacto con el antibiótico, los linfocitos se activan y comienzan a proliferar. Este proceso de proliferación conduce a la dilución secuencial del fluoróforo cuya intensidad de emisión puede medirse, de modo que las generaciones celulares pueden cuantificarse mediante citometría de flujo. [62]

Se ha demostrado que en general es más sensible que las ST para las NIR y presenta una sensibilidad y especificidad que oscilan entre el 58-88% y entre el 85-100%, respectivamente cuando se trata de antibióticos BLCs. [62]

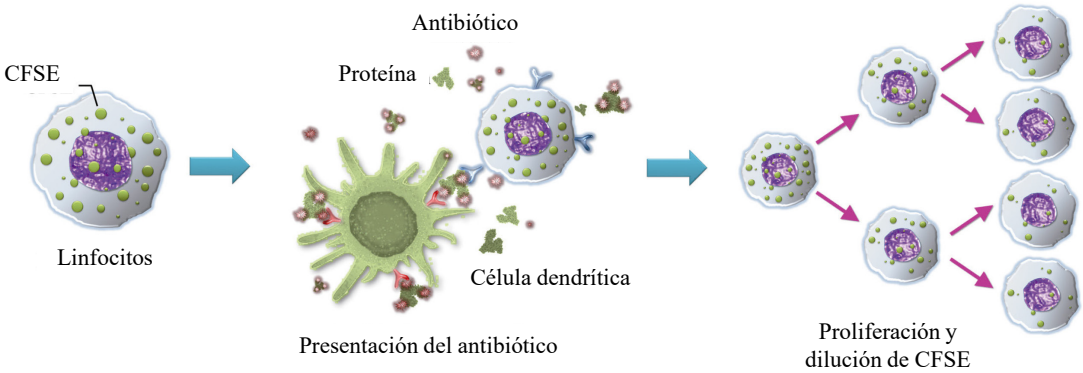

Figura 9: Esquema de la prueba de transformación de linfocitos.[61]

1.2.3.3. Prueba de liberación de histamina.

Esta prueba se basa en la detección de histamina liberada por parte de los basófilos presentes en la sangre tras la estimulación con el fármaco sospechoso. El procedimiento optimizado consiste en la incubación de sangre heparinizada en placas de microfibra de vidrio y la estimulación con el antibiótico de interés, seguida de la detección de la liberación de histamina mediante técnicas fluorométricas.[61] Esta prueba se ha utilizado para el diagnóstico de reacciones alérgicas a varios alérgenos, pero raramente fármacos.[64] Recientemente, también se ha utilizado para la evaluación de alergia a clavulánico, mostrando una sensibilidad del 55% y una especificidad del 85%. A pesar de los prometedores resultados, es necesario estandarizar esta metodología como prueba diagnóstica para las reacciones alérgicas a antibióticos BLCs. [61]

1.2.3.4. Prueba de activación de basófilos

La prueba de activación de basófilos (Figura 10) se basa en la medida de expresión de los marcadores de activación en la superficie de los basófilos, como resultado de la unión de la IgE a los receptores de alta afinidad tras la estimulación del alérgeno.[65] Los marcadores más utilizados para determinar dicha activación son CD63 y CD203c. El CD63 se expresa en la membrana de las vesículas que contienen histamina y que se muestra en gran medida en la superficie de los basófilos tras la desgranulación.[66] El CD203c también se regula al alza tras la activación de los basófilos. [67,68]

Esta prueba se puede usar para evaluar reacciones inmediatas frente a antibióticos BLCs, especialmente para aquellos para los que no existe un inmunoensayo, como el ácido clavulánico y el cefazolin. Su sensibilidad está en torno al 50% y 70%, y su especificidad entre el 90% y 95%.[61]

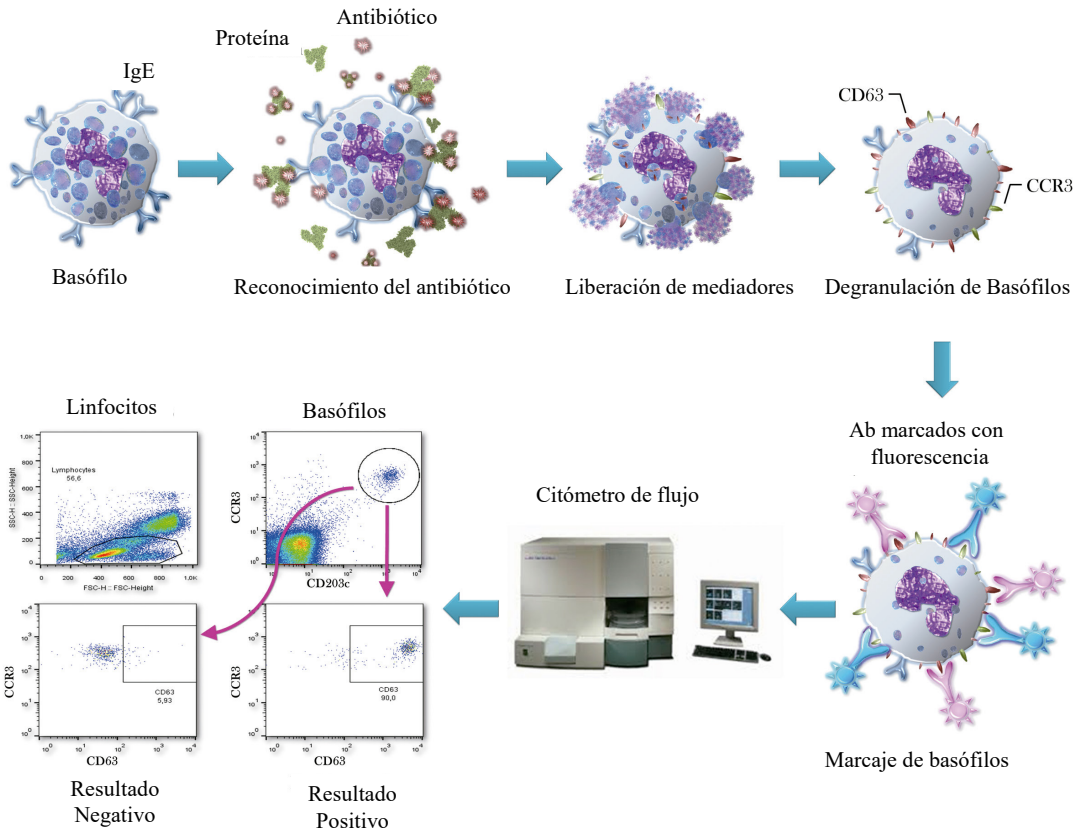

Figura 10: Esquema de la prueba de activación de basófilos.[61]

1.2.3.5. Inmunoensayos

Los inmunoensayos constituyen el método más empleado para el diagnóstico *in vitro* de alergias y se basan en la determinación de nivel de IgE específica (sIgE) al fármaco presente en suero o plasma.[69] En la alergia a fármacos, la cuantificación de la sIgE se basa en la detección de un complejo carrier-fármaco(hapteno)-anticuerpo. En general, este conjugado carrier-hapteno se acopla a una fase sólida, que se incuba con el suero del paciente. La cantidad de sIgE unida se detecta utilizando anticuerpos anti-IgE marcados con un radioisótopo (RIA), o con enzimas para la detección colorimétrica o fluorescente.[62]

El primer inmunoensayo desarrollado fue la prueba de radioalergoabsorbancia, o RAST, descrita inicialmente en 1967 para la detección de anticuerpos específicos de alérgenos.[70] En su forma original, el RAST utilizaba un alérgeno acoplado covalentemente a Sephadex activado con bromuro de cianógeno.[71] Los anticuerpos específicos del alérgeno que reconocían el antígeno fijado en la fase sólida eran detectados posteriormente con un anticuerpo secundario anti-IgE humana marcado con un radioisótopo (^{125}I). Este ensayo inicial evolucionó a un ensayo más sencillo utilizando

como fase sólida un disco de papel. Posteriormente, en 1989, se lanzó el sistema Pharmacia CAP, conocido como "RAST de segunda generación" que sustituyó al disco de papel por una nueva fase sólida, ImmunoCAP (ICAP), consistente en un polímero hidrófilo configurado en una pequeña cápsula. Esta fase sólida presenta una capacidad de unión más elevada tanto para el acoplamiento covalente de antígenos como para las interacciones con anticuerpos específicos, en comparación con el papel de celulosa convencional.[72] Esta mejora de las prestaciones del ensayo, junto con el uso de un método de detección enzimática por fluorescencia, considerado más seguro que la detección de radiactividad, condujo al desarrollo de ensayos automatizados, como el Pharmacia ImmunoCAP System FEIA (Figura 11).[73] Aunque es el método comercial más utilizado, ICAP solo está disponible para penicilina G, penicilina V, amoxicilina, ampicilina y cefaclor. Además, su sensibilidad es bastante baja y variable (0-50%)[74,75]. Estudios recientes han revelado que la sensibilidad diagnóstica puede mejorarse alcanzando 0.1 IU/mL como umbral de detección, sin embargo se produce una disminución de la selectividad dando lugar a falsos positivos.[54]

Aunque estos inmunoensayos suelen tener una sensibilidad insuficiente, ésta puede variar en función de las manifestaciones clínicas de la reacción investigada, del fármaco implicado y del intervalo de tiempo entre la aparición de la reacción y la realización de la prueba.[76] Otros factores están relacionados con la unión del fármaco a la fase sólida, la molécula carrier utilizada como parte del determinante antigénico y la densidad de hapteno en el conjugado.

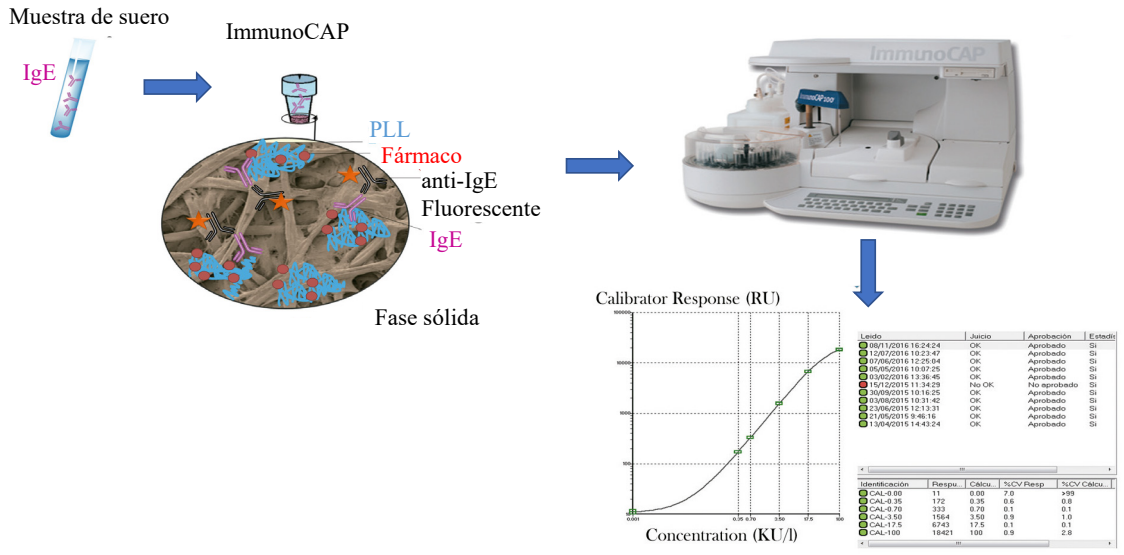

Figura 11: Ilustración del proceso de determinación de sIgE mediante ICAP.[61]

Además de ImmunoCAP, otros inmunoensayos han ido apareciendo en el mercado basados en la determinación de sIgE en suero. En la Tabla 1 se muestran las principales características de los métodos más utilizados en la actualidad para el diagnóstico de alergia.

Tabla 1: Sistemas utilizados en clínica para la determinación de sIgE.

Nombre	Detección	Vol (µl)	Tiempo (min)	LOD (IU/mL)	SB*	EP*	Ref
ImmunoCAP	Fluorometría	40	100	0.10	30	90	47,59,77
Immulite	Luminiscencia	50	65	0.10	30	100	77,78
ImmunoCAP ISAC	Fluorometría	30	240	0.30	50	100	47
ALEX	Colorimetría	100	190	0.30	--	--	47
Euroline	Colorimetría	100-400	130	0.35	--	--	47,77
Allerg-O-Liq	Colorimetría	100	180	0.35	50	100	77,79
Polycheck	Colorimetría	200	150	0.35	40	80	77
Luminex	Fluorometría	20	360	0.35	--	--	77
NOVEOS Hycor	Colorimetría	4	95	0.20	50	100	80,81
IVD Capsule Aeroallergens	Fluorometría	50	60	0.70	--	--	82
ALFA	Colorimetría	30	60	0.35	40	100	83,84

*SB: sensibilidad diagnóstica. EP: especificidad diagnóstica. La especificidad y la sensibilidad se detalla exclusivamente en aquellos métodos que diagnostican alergia a antibióticos BLCs

Aunque los distintos métodos están basados en el mismo principio difieren en el grado de automatización, la fase sólida, la forma de unión de los alérgenos, el antígeno utilizado, el modo de detección de la señal, el volumen de muestra necesario y el formato de ensayo. Todas estas diferencias han llevado a los inmunólogos y a la comunidad científica a preguntarse si los métodos son comparables entre sí y la evidencia científica ha puesto de manifiesto que los resultados no lo son.[76,85,86] Aunque en algunos casos haya cierta correlación de resultados, al no existir unidades referidas a un estándar específico común, los resultados no son comparables mediante el uso de factores de conversión.[47]

1.2.4. Factores clave en el desarrollo de inmunoensayos para el diagnóstico de alergias a antibióticos BLCs.

La determinación de la sIgE sérica en una muestra de suero es el método diagnóstico *in vitro* para alergias más extendido. Como se ha comentado en el apartado anterior, estas técnicas se basan en la unión de un determinado alérgeno a una fase sólida o líquida y una

posterior incubación del suero. Las IgE no específicas se eliminan mediante lavado y las IgE específicas unidas al alérgeno son detectadas por un anticuerpo anti-IgE marcado.[47]

Entre los parámetros de un inmunoensayo la superficie, el antígeno y el sistema de calibración son clave en la determinación de IgE.

1.2.4.1. Superficie

Como se ha comentado anteriormente, el primer paso a la hora de desarrollar un inmunoensayo es la unión del antígeno o anticuerpo a una superficie. La inmovilización del antígeno puede realizarse mediante adsorción o mediante anclaje covalente.[87] En los ensayos en fase sólida, la superficie de unión puede estar en tubos, micropocillos de placa ELISA o partículas magnéticas.[88] En la mayoría de los inmunoensayos en fase sólida, la proteína se une a la superficie de un polímero plástico, normalmente poliestireno[87], aunque también se han descrito inmunoensayos en formato microarray en superficies de policarbonato, como un DVD o Blu-ray.[89]

En los últimos años han aparecido nuevas fases sólidas como el CAP desarrollado por Phadia que consiste en un gel encerrado en una cápsula y que mejora la exposición del antígeno.[90] Otros métodos *in vitro* actualmente en el mercado como ALFA utilizan discos de celulosa[84], Immulite o Hycor utilizan partículas de poliestireno funcionalizadas[77,80], y otras como Polycheck utilizan membranas[77].

1.2.4.2. Antígeno

Los antibióticos BLCs sufren un proceso de haptenización dando lugar a un aducto fármaco-proteína que termina desencadenando la respuesta alérgica. Es decir, este conjugado es reconocido por la sIgE y por tanto se debe utilizar como determinante antigénico en los inmunoensayos. Sin embargo, todavía no se conocen en profundidad y detalle los mecanismos de haptenización *in vivo*.

La albúmina sérica humana (HSA) es la proteína más abundante del plasma. Presenta una extraordinaria capacidad de unión a ligando y se ha demostrado que desempeña un papel crucial como portadora de compuestos endógenos y exógenos[2], incluidos varios fármacos. Basándose en esta evidencia, la HSA se consideró tradicionalmente la principal proteína diana en el proceso de haptenización de penicilinas y la mayoría de los estudios se han centrado en la caracterización de los aductos penicilloil-HSA. Sin embargo, más adelante se ha descubierto que el grado de conjugación de la HSA depende del fármaco,

la concentración de este y del tiempo de incubación, siendo esto clave en la sensibilidad de los métodos.

Otras proteínas séricas como la transferrina[91] y las inmunoglobulinas[92,93], y no séricas[94,95] podrían estar implicadas en el proceso de haptenización y en la inducción de una respuesta inmunitaria; sin embargo, se sabe muy poco sobre su naturaleza o su papel en el desarrollo de una reacción de hipersensibilidad. De hecho, recientemente se han descrito nuevos antígenos que utilizan la histona H1 como proteína de conjugación. Estos conjugados muestran muy buenos resultados debido a su alto grado de conjugación, así como por la buena disposición del hapteno para que sea reconocido por la IgE.[95]

Hay estudios de mejora de la sensibilidad de estas pruebas que se han centrado en el uso de estructuras dendriméricas como moléculas portadoras de antibióticos BLCs, así como nuevas estrategias para la funcionalización de las fases sólidas, que permitirían una mayor densidad de determinante antigénico y, por tanto, se prevé una mayor sensibilidad de la prueba.[2]

Sin embargo, la limitación que existe a la hora de evaluar nuevos determinantes antigénicos es la necesidad de disponer de calibradores o muestras procedentes de pacientes alérgicos. Hasta ahora, solamente se puede saber si un nuevo determinante antigénico concluirá en un ensayo más sensible utilizando la sIgE presente en el suero de un paciente alérgico.

1.2.4.3. Calibración

Actualmente existen inmunoensayos cualitativos, que sólo generan resultados positivos o negativos; semicuantitativos, donde se clasifica en diferentes clases, por ejemplo, de la I a VI, determinando así un rango de concentración; y cuantitativos, los que proporcionan un valor de concentración de sIgE.[96]

La cuantificación hace necesario disponer de una curva de calibración obtenida a partir de patrones o materiales de referencia. Dado que hasta ahora no existen estándares de calibración específicos aceptados internacionalmente para las pruebas de sIgE se recurre a una calibración heteróloga[97]. Esta consiste en interpolar las señales medidas en el ensayo para la determinación de sIgE en una curva de IgE total obtenida utilizando los estándares oficiales de la OMS (actualmente OMS 11/234). Los resultados se presentan en unidades kU/L de IgE, donde una unidad equivale a 2.4 ng/mL de IgE.[98]

El umbral de detección más bajo para la sIgE es, por lo general, de 0.35 kU_A/L. Este límite se establece mediante el análisis de curvas ROC con muestras de pacientes y controles, y con él se consigue determinar el punto de corte en el que se alcanza la sensibilidad y especificidad más alta. Estudios recientes contemplan la disminución del umbral de detección a 0.10 kU_A/L, sin embargo, en las pruebas realizadas con los métodos actuales se observa una pérdida de especificidad diagnóstica del 95% al 54%.[54,99]

A diferencia del inmunoensayo para la determinación de sIgE específica, para la obtención de esta curva de IgE total se realiza un inmunoensayo tipo sándwich donde, en la fase sólida, el antígeno se sustituye por un anticuerpo de captura (Figura 12). Como parte de este proceso, se asume una afinidad comparable entre el alérgeno (determinante antigénico) y la IgE específica, y entre los anticuerpos anti-IgE de captura utilizados para preparar la curva de referencia y la IgE total.[97] Pero estudios recientes han demostrado que la constante de afinidad aparente depende del antígeno y que la calibración heteróloga puede afectar a la precisión del método, siendo los métodos de calibración homólogos más precisos y robustos para la determinación de sIgE.[100]

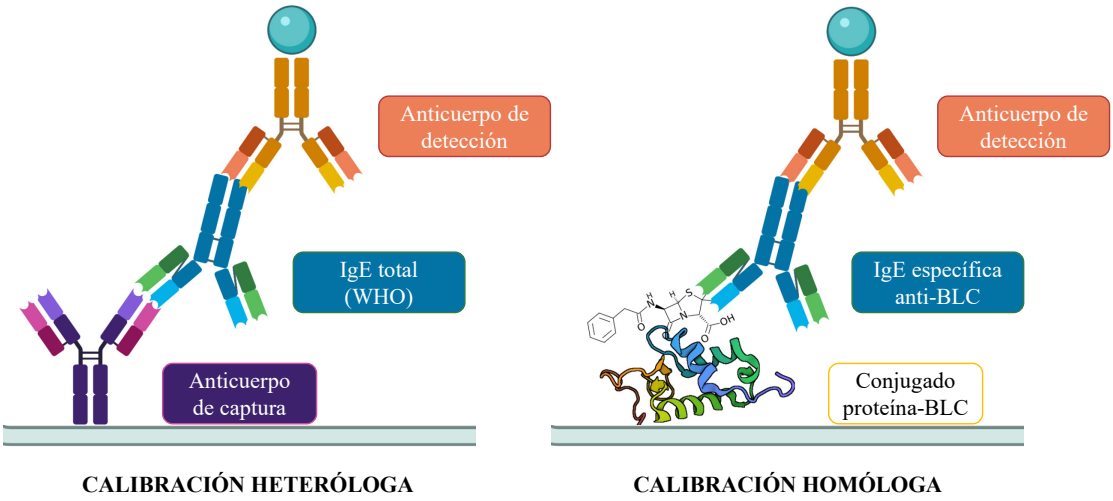

Figura 12: Esquema del ensayo con calibración heteróloga (WHO) y homóloga (IgE específica).

El primer intento de aproximación a los estándares sintéticos ha consistido en la obtención de quimeras humanizadas. Estos anticuerpos se obtienen mediante la unión del Fab de un anticuerpo específico obtenido por inmunización animal y la Fc humana.[101,102,103] Estas moléculas quiméricas permiten realizar una calibración homóloga y ser utilizadas como controles positivos en la puesta a punto de nuevos ensayos. Sin embargo, la principal desventaja asociada a este tipo de moléculas como reactivo es que

su origen animal hace que su producción sea limitada y poco reproducible, lo que impide tener un producto bien definido y caracterizado en las distintas tandas de producción.

1.2.5. Validación de ensayos

La validación de los métodos de diagnóstico es un trabajo que se realiza a nivel internacional en pruebas "*round-robin*". Esta metodología implica la coordinación de distintos grupos científicos internacionales para evaluar el nuevo método, examinarlo y asegurar que cumple los estándares de calidad. Los ensayos de aptitud se realizan de acuerdo con las normas ISO 13528 e ISO/IEC 17043 y se verifican la reproducibilidad, la sensibilidad y especificidad analítica y diagnóstica.[104]

Analíticamente, se evalúa la especificidad por el analito de interés, sIgE en este caso. Si ésta es alta se traduce en una mejora de la sensibilidad, es decir, un límite de detección bajo. Sin embargo, como se mencionaba anteriormente, es imposible evaluar la sensibilidad analítica del ensayo sin muestras procedentes de pacientes alérgicos que contienen el analito de interés. Dicha evaluación, en el caso de las alergias a antibióticos BLCs, es aún más complicada, ya que las muestras son escasas, la concentración de sIgE suele ser bastante baja (<10 IU/mL) en comparación con alergias alimentarias o ambientales y no se dispone de ensayos para cuantificar el nivel de sIgE de la mayoría de los antibióticos prescritos.

En el ámbito diagnóstico, la sensibilidad se refiere a la probabilidad de que la prueba identifique como alérgico a aquel que efectivamente lo es, mientras que la especificidad indica la probabilidad de etiquetar como no alérgico a alguien que realmente no es alérgico. Como se puede observar, están relacionadas con los síntomas del paciente y por lo tanto es estrictamente necesario tener muestras procedentes de pacientes de las que se conozca su historial clínico.

Evaluar estos parámetros para cada ensayo implica realizar una validación clínica exhaustiva e interlaboratorio de la nueva técnica de diagnóstico[105], es decir, hay que ejecutar un estudio que tenga en cuenta la realidad poblacional para verificar que el nuevo método de diagnóstico cumple con los requisitos necesarios en cuanto a sensibilidad y especificidad analítica.[47] Sin embargo, hasta la fecha, la legislación europea de productos sanitarios no exige una validación previa a la comercialización de ensayos clínicos realizados con sueros procedentes de pacientes.[106] En Europa, el único requisito para la comercialización de productos sanitarios es la obtención del sello CE. Para esto se

requiere al fabricante presentar un estudio de comparación con otro método y demostrar que es reproducible en el tiempo.[47] Es por ello que en el caso de los métodos de diagnóstico *in vitro* de alergia, actualmente no hay ningún método validado y se utiliza ImmunoCAP como método de referencia por ser el método más extendido. Sin embargo, estudios comparativos han puesto de manifiesto que al no existir unidades referidas a un estándar común, lo resultados entre distintos métodos no son comparables.[107]

En definitiva, hay una demanda de realizar una validación completa de este tipo de inmunoensayos basados en la determinación de IgE, ya que es el biomarcador con más peso en el diagnóstico de hipersensibilidad de tipo I.

1.3. INMUNOGLOBULINAS

Los anticuerpos o inmunoglobulinas son producto de la evolución del sistema inmune adaptativo ya que son capaces de reconocer gran variabilidad de antígenos de forma selectiva. Esta propiedad de unión ajustable es posible gracias a un complejo conjunto de mecanismos que alteran el ADN de los linfocitos B, las células encargadas de su producción. Las inmunoglobulinas se presentan inicialmente como receptores de membrana celulares y frente al reconocimiento de un patógeno o antígeno las células proliferan y se diferencian en plasmocitos que secretan anticuerpos con la misma selectividad de unión al antígeno que presentaba su receptor de membrana.[108]

En los humanos se reconocen 5 clases distintas de inmunoglobulinas: Inmunoglobulina G (IgG), M (IgM), A (IgA), D (IgD) y E (IgE). Cada clase (isotipo) se distingue por la secuencia de aminoácidos que forma la región constante de la cadena pesada. Esa secuencia única les confiere, por tanto, diferentes propiedades estructurales y funcionales. Dentro de las inmunoglobulinas, la de tipo E está implicada en las reacciones de hipersensibilidad, lo que la convierte en el biomarcador más utilizado en alergia.

Sin embargo, estas no son las únicas inmunoglobulinas que existen en el reino animal, ya que algunas especies como los camélidos o algunos peces cartilaginosos presentan anticuerpos que carecen de cadena ligera, denominados anticuerpos de cadena pesada.

1.3.1. Inmunoglobulina tipo E

La IgE es la inmunoglobulina responsable de las reacciones de hipersensibilidad, lo que la convierte en el biomarcador más importantes a la hora de diagnosticar este tipo de reacciones adversas. Posee una masa molecular de 188 kDa[109] y es una proteína heterotetramérica formada por dos cadenas pesadas (identificadas por el sufijo H) y dos cadenas ligeras (identificadas por el sufijo L).

Cada cadena ligera, asignada al tipo κ o λ, forma un heterodímero con una cadena pesada y dos de esos heterodímeros resultantes, que están unidos mediante enlaces disulfuro (L-H así como H-H), representan la inmunoglobulina intacta. Dentro de la molécula, cada cadena ligera se compone generalmente de un dominio variable N-terminal (V_L) así como de un dominio constante (ya sea $C_L\kappa$ o $C_L\lambda$), mientras que cada una de las cadenas pesadas dominio variable N-terminal (V_H) seguido, en el caso de la IgE, de cuatro regiones constantes (C_H1-C_H4).[108] Desde una perspectiva funcional, el

anticuerpo tiene dos fragmentos idénticos responsables de la unión al antígeno (Fab) y el fragmento cristalizable (Fc) que media las funciones efectoras (Figura 13).[110,111]

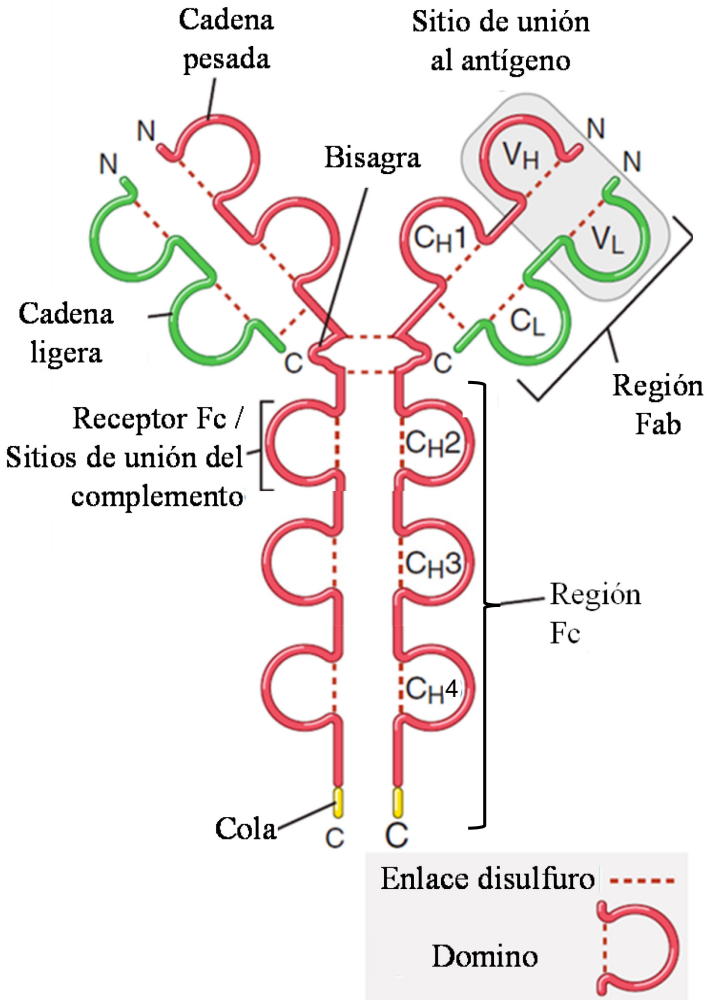

Figura 13: Estructura de la IgE.[112]

Dentro del dominio de la región Fab, V_H y V_L albergan el sitio de unión al antígeno, es decir, el parátopo. Este parátopo está compuesto por tres bucles hipervariables, denominados regiones determinantes de complementariedad (CDR), y ofrecen una extraordinaria variabilidad con respecto a la longitud de los bucles, así como a la composición de aminoácidos.[113]

Son estos CDRs lo que permiten que los anticuerpos sean capaces de reconocer cualquier molécula extraña que ingresa al organismo con una alta especificidad y afinidad. La amplia diversidad de reconocimiento se debe a los procesos de recombinación somática (Figura 14), que ocurren durante el desarrollo del linfocito B y en la etapa de maduración de la afinidad. [114]

Dado que los anticuerpos contienen dos brazos Fab idénticos, la unión al antígeno es bivalente y monoespecífica por naturaleza. La unión bivalente simultánea de dos dianas idénticas es posible gracias a la región bisagra, que conecta ambos brazos Fab y la parte Fc, y que aporta suficiente flexibilidad.[115]

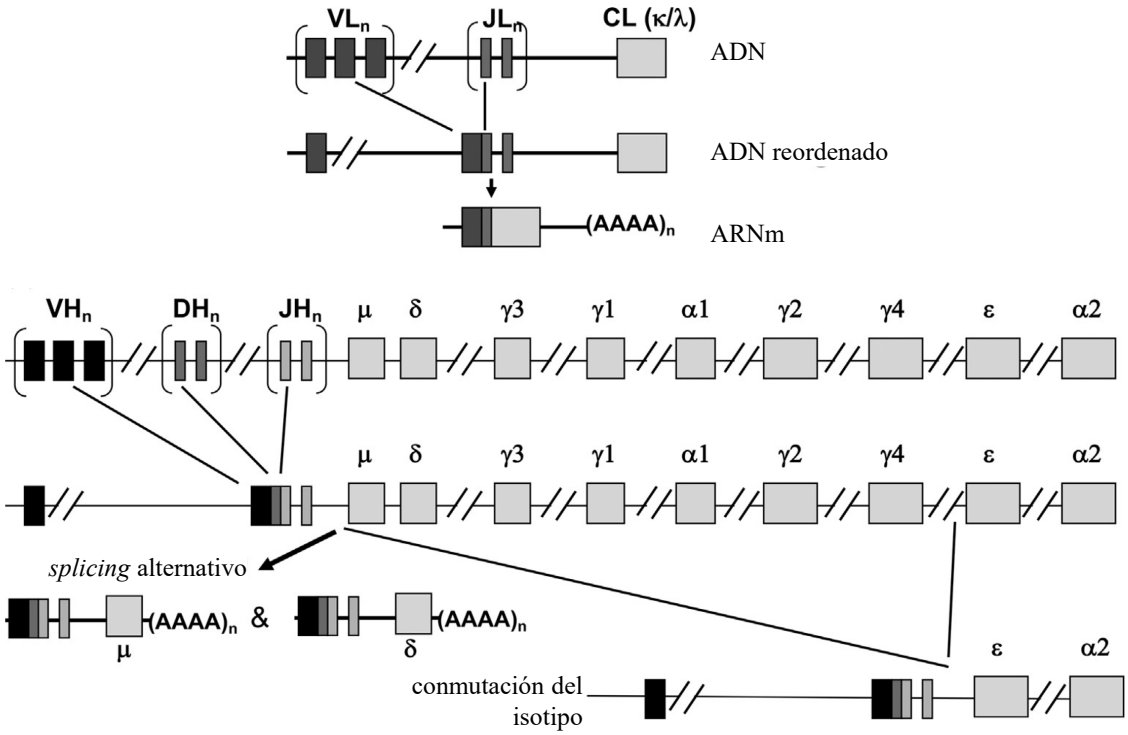

Figura 14: Proceso de mutación y recombinación somática.[116]

1.3.2. Anticuerpos de cadena pesada

En el año 1993, Hammer-Casterman y colaboradores, reportaron la existencia de anticuerpos homodiméricos carentes de cadena ligera en el suero de camélidos[117], denominados por lo tanto anticuerpos de cadena pesada (HcAbs). En este tipo de anticuerpos el sitio de unión a antígeno está conformando únicamente por la región N-terminal de la cadena pesada y es denominado VHH (para diferenciarlo del dominio variable VH de las inmunoglobulinas convencionales), y su expresión recombinante es denominado "nanobody" o nanoanticuerpo. Otra particularidad de los HcAbs es que carecen del dominio C_H1, y por lo tanto el VHH está directamente unido a la región bisagra (Figura 15).[117]

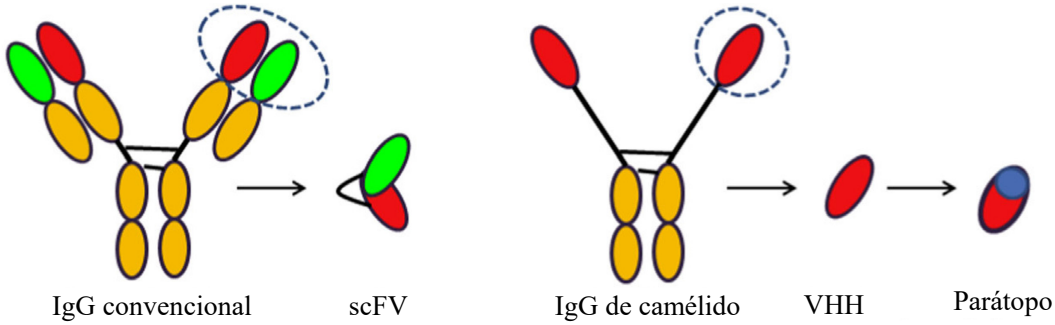

Figura 15: Diferencias entre una IgG convencional y una IgG de camélido.[118]

El VHH adopta una estructura de hojas β al igual que en el dominio VH, que se conforma de 4 hojas-β y 5 hojas-β conectadas a través de 3 CDRs y por un puente disulfuro conservado.[119] De esta forma los CDRs en la región N-terminal generan una región continua que conforma el sitio de reconocimiento a antígenos. Sin embargo, el alineamiento de las secuencias de aminoácidos de los VHH y VH, demostraron que existen importantes diferencias. Residuos hidrofóbicos altamente conservados que normalmente interaccionan con el dominio V_L en los anticuerpos convencionales, en los VHH son sustituidos por residuos más hidrofílicos y/o de menor tamaño.[120] Estas modificaciones son responsables de generar un dominio VHH más soluble y estable, reduciendo la tendencia a agregarse.

Debido a que la diversidad combinatoria generada por el apareamiento de las regiones VH/VL no está presente, es esperable que los camélidos generaran un repertorio de HcAbs muy limitado. Sin embargo, lo cierto es que los VHH presentan ciertas características distintivas que son responsables de aumentar el repertorio estructural y generar más diversidad de secuencias en un sitio de unión provisto con la mitad de CDRs en comparación a los anticuerpos convencionales. [121,122] En esta dirección, se ha observado que el CDR1 y en especial CDR3 son más largos en los VHH con respecto a los VH, lo que permite incrementar la superficie de interacción con el antígeno. Así mismo, en la mayoría de los casos se observa que la estructura del CDR3 se pliega reduciendo la exposición de residuos hidrofóbicos al disolvente. Los extensos CDRs hacen que resulten particularmente eficientes para acceder a epítopos cóncavos que no siempre son accesibles a los anticuerpos convencionales, lo que permite que los nanoanticuerpos sean capaces de inhibir enzimas o de unirse a epítopos crípticos[123], siendo ésta una de las aplicaciones más prometedoras de los mismos.

1.3.3. El uso de anticuerpos en clínica

Los experimentos para utilizar los anticuerpos policlonales (pAb) como terapia existen desde hace más de un siglo[124], sin embargo, la primera descripción de anticuerpo monoclonal (mAb) creado en el laboratorio no se publicó hasta 1970, y no fue has 1975 cuando se generó el primer mAb para su uso en humanos.[125]

Los primeros mAbs se generaron utilizando proteínas murinas (procedentes de ratones), pero al ser inmunogénicos no eran bien tolerados por los humanos. Por lo tanto, los mecanismos de producción tuvieron que ser reevaluados y finalmente modificados por el desarrollo de la tecnología, lo que permitió obtener anticuerpos totalmente humanizados.[126]

Debido a su alto grado de selectividad, los mAbs permiten detectar dianas muy precisas que pueden determinar cambios celulares muy diversos; además, dependiendo de la región Fc en cuestión, pueden diseñarse para facilitar distintos tipos de respuestas efectoras. Todo esto abre la puerta a una amplia gama de posibles aplicaciones en medicina.[127] Como resultado hasta la fecha, 106 anticuerpos monoclonales y conjugados de anticuerpos han sido aprobados por la EMA y/o la FDA, 87 anticuerpos están en fase clínica 2 o 3 y 122 están en fase 1 o se han presentado como nuevo fármaco en investigación.[128] La mayoría de los anticuerpos aprobados son para el tratamiento de enfermedades autoinmunes y de cáncer.

Las ventas anuales de anticuerpos terapéuticos superaron los 98.000 millones de dólares en 2017 y se prevé que el mercado alcance entre 137.000 y 200.000 millones de dólares en 2022.[129]

Los modos de acción de los anticuerpos terapéuticos son numerosos e incluyen la neutralización de sustancias, por ejemplo, citoquinas como el factor alfa de necrosis tumoral (TNF-a)[130] o toxinas[131], la unión de células humanas y la modulación del sistema inmunitario del huésped[132], el bloqueo de receptores que se sobreexpresan en las células cancerosas como el receptor del factor de crecimiento epidérmico[133] o combinaciones de estos modos de acción.[134]

1.3.3.1. Clasificación y tipos de mAbs

Según su estructura (Figura 16), los mAbs se pueden clasificar en murinos, quiméricos, humanizados y humanos en base a su composición.

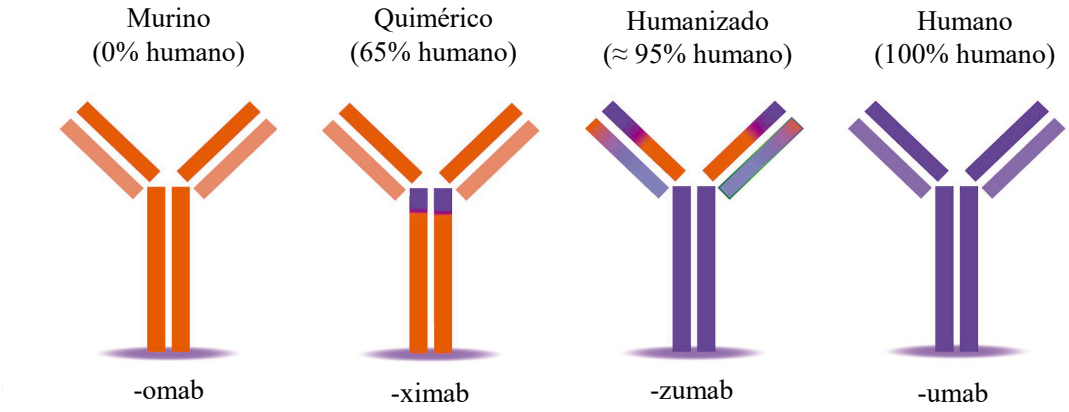

Figura 16: Clasificación de los mAbs.

El primer mAb que se descubrió y reprodujo fue el anticuerpo monoclonal murino. Este tipo de mAb surge a partir de los linfocitos B del bazo de un ratón y luego se fusiona con una línea celular de mieloma inmortal.[125] Todos estos mAbs se identifican con un nombre que termina en -omab. Sin embargo, no se pueden utilizar en terapia en humanos ya que son inmunogénicos y a menudo dan lugar a anticuerpos contra el fármaco.[126]

En el caso de los mAbs quiméricos, utilizan la región variable específica del antígeno murino, pero el resto de las cadenas pesadas y ligeras son humanas.[127] Esto se logró mediante técnicas de ingeniería genética que dieron como resultado mAbs que son en su composición aproximadamente un 65% humanos y un 35% murinos. Los mAbs quiméricos se identifican con nombres que terminan en -ximab. En comparación con sus homólogos murinos muestran una inmunogenicidad reducida, no obstante, la predisposición de los mAbs quiméricos a inducir anticuerpos antifármaco es elevada.[135]

Los mABs humanizados se crean mediante la inserción de los CDRs murinos de las cadenas pesadas y ligeras en un marco de anticuerpo humano.[125,126] Esto da lugar a moléculas que son aproximadamente un 95% de origen humano. De esta forma, se reduce la inmunogenicidad. Sin embargo, el proceso de producción de estas moléculas es arduo y tiene limitaciones, como la disponibilidad del antígeno a la hora de inmunizar a los ratones.[136] Estos mAbs se identifican con nombres que terminan en -zumab.[126]

Y por último, con el desarrollo de nuevas metodologías, como Phage Display, se han podido obtener mAbs totalmente de origen humano. Estos se crean utilizando genes de inmunoglobulinas humanas.[135] Estos mAbs son menos inmunogénicos y, por tanto, mejor tolerados en comparación con las otras clases de mAbs. Estos mAbs se identifican con nombres que terminan en -umab.[126]

1.3.3.2. Anticuerpos como terapia de alergia

La última década ha sido testigo de una transformación en los enfoques para tratar las enfermedades alérgicas con el desarrollo, aprobación y uso creciente de productos biológicos. El asma fue la primera enfermedad alérgica a la que se dirigieron los anticuerpos para reducir la respuesta alérgica en las vías respiratorias.[137]

Omalizumab fue el primer biológico que se aprobó a nivel mundial para el tratamiento de asma alérgica[138] y posteriormente se extendió su uso para el tratamiento de urticaria[139]. Se trata de una IgG1κ monoclonal humanizada que se une específicamente al dominio Cε3 de la región Fc de la IgE humana, uniéndose así a la IgE libre[140]. Esta unión impide que la IgE se una a los receptores FceRIa de basófilos y mastocitos, y, por lo tanto, la posterior activación de las células efectoras[141]. Posteriormente se han ido desarrollado nuevos biológicos anti-IgE con mayor afinidad y que, en las primeras fases de ensayo clínico, están mostrando buenos resultados en el tratamiento de enfermedades alérgicas como Ligelizumab[142], Quilizumab (Genentech)[143] y XmAb7195 (Xencor)[144].

Junto con estos biológicos, también se han desarrollado otros con dianas relacionadas con las reacciones alérgicas como Benralizumab (Fasenra; AstraZeneca), Mepolizumab (Nucala; GalxoSmithKline), Reslizumab (Cinqair) dirigidos contra IL-5 y con resultados prometedores en el tratamiento contra el asma.[141]

1.4. PHAGE DISPLAY

Los anticuerpos son herramientas básicas en investigación aplicada[145], diagnóstico[146] y terapia[147]. Inicialmente, los anticuerpos usados eran policlonales que se obtienen a partir del suero sanguíneo de animales inmunizados. Posteriormente, las diferentes tecnologías desarrolladas han permitido hacer avances importantes, como la tecnología del hibridoma, que permitió la producción de anticuerpos monoclonales de ratón.[148] Sin embargo, esta tecnología tiene ciertas limitaciones como que a pesar de ser monoclonales, estos anticuerpos no siempre son monoespecíficos[149]. A esto hay que añadir la inestabilidad de las líneas celulares que producen el anticuerpo[150], la restricción del sistema inmunitario que impide la generación de inmunoglobulinas contra antígenos altamente conservados o propios y, lo más importante, no permite generar directamente anticuerpos totalmente humanos.[151] La solución para superar todas estas limitaciones es la obtención de anticuerpos recombinantes. Estos son anticuerpos monoclonales obtenidos a partir de genes sintéticos y producidos mediante metodología *in vitro*. En la tabla 2 se recogen las principales ventajas y desventajas de los pAb, los mAb y los anticuerpos recombinantes.

Tabla 2: Comparación de los diferentes tipos de anticuerpos.[152]

	Policlonales	**Monoclonales**	**Recombinantes**
Tiempo de producción	Corto (3-4 meses)	Largo (hasta un año)	Moderadamente largo (4-8 meses)
Coste de producción	Bajo	De moderado a alto	Intermedio
Facilidad de producción	Muy fácil	Laborioso	Fácil
Diana	Dianas inmunogénicas	Dianas inmunogénicas	Sin limitaciones
Estabilidad	Alta	Moderada	Depende del formato
Reproducibilidad	Limitada	Prácticamente reproducible	Totalmente reproducible
Especificidad	Moderada	Alta	Alta
Sensibilidad	Variable	Alta	Alta
Modificaciones estructurales	No es posible	No es posible	Posible
Disponibilidad	Comercialmente disponible	Comercialmente disponible	Disponibilidad comercial limitada

Una metodología que permitió superar dichas limitaciones y generar anticuerpos recombinantes monoespecíficos es la metodología Phage Display basada en el trabajo de Georg P. Smith sobre fagos filamentosos que infectan a *E. coli.*[153] Se desarrolló en paralelo en 1990 en el German Cancer Research Center en Heidelberg (Alemania), en el Medical Research Council Laboratory of Molecular Biology en Cambridge (Reino Unido) y en el Scripps Research Institute en La Jolla (Estados Unidos).[154–157] El bacteriófago más ampliamente utilizado es el M13, perteneciente a un grupo de fagos filamentosos colectivamente denominados Ff. Este grupo de fagos es específico para las células *E. coli* que portan el plásmido codificante del pili F, ya que este pili de conjugación es la puerta de entrada que utilizan estos fagos para realizar la infección.

El fago M13 no es fago lítico, sino que establece una infección crónica dentro de su huésped donde continuamente produce nuevos fagos. El genoma del fago consiste en ADN circular de simple hebra (ssADN) de 6407 pb, que codifica para 11 proteínas diferentes, 5 proteínas de la cápside y el resto son proteínas involucradas en la replicación y ensamblaje de la partícula viral. Una vez infectada la bacteria, el fago lleva adelante su ciclo de replicación en su interior y las partículas virales comienzan a ensamblarse sobre la superficie de la membrana bacteriana. Durante este proceso la molécula de ADN que codifica para las proteínas del fago es encapsulada dentro de la cápside, estableciéndose un vínculo físico entre genotipo y fenotipo.

Para llevar a cabo el Phage Display es necesario disponer de una biblioteca de péptidos expresados en fagos que abarque toda la variabilidad de anticuerpos generados. En la construcción de dicha biblioteca se generan modificaciones genéticas para promover la expresión de proteínas de cápside fusionadas en sus extremos (generalmente N-terminal) a fragmentos de anticuerpos o péptidos que determinarán el fenotipo de cada clon. De las proteínas de la cápside, la pIII (5 copias por partícula) es ampliamente utilizada como proteína de fusión para la construcción de bibliotecas de anticuerpos y péptidos recombinantes.[158] La fusión del péptido o anticuerpo a la proteína en cuestión puede estar codificada en el propio genoma del fago o en un vector separado, denominado fagémido, que puede ser encapsulado (tal como lo hace el genoma del fago) con la ayuda de un fago helper. Los vectores fagémidos son plásmidos que codifican la fusión del péptido/anticuerpo a la proteína del fago, así como un gen de resistencia a antibiótico para favorecer su selección. El resto de las proteínas que dan lugar a la replicación y empaquetamiento del fagémido son provistas a través de la sobre infección con el fago

helper. Dicho fago tiene su origen de replicación "f1 ori" modificado y defectuoso lo que hace que su propia replicación y su empaquetamiento sean poco eficientes, lo que promueve un empaquetamiento preferencial por parte del fagémido durante el proceso de infección.[159] (Figura 17)

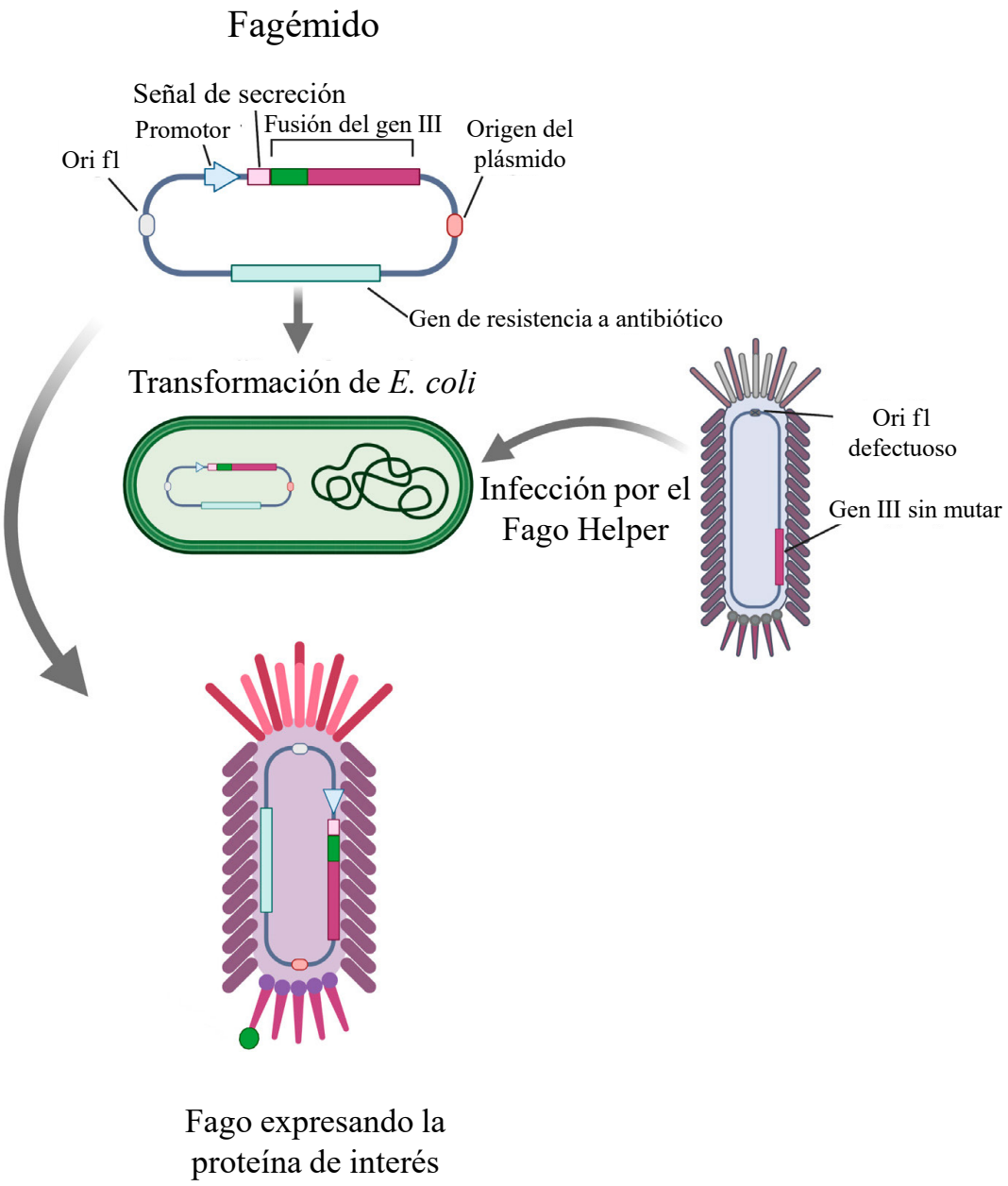

Figura 17: Obtención del fago expresando la proteína de interés.[160]

Debido a su naturaleza compleja, el Phage Display de anticuerpos de longitud completa suele ser difícil de lograr. En consecuencia, los formatos de anticuerpos más utilizados son el fragmento de unión a antígeno (Fabs)[161] o el fragmento variable de cadena única (scFv).[162] Los scFv sólo constan de los dominios VH y VL conectados por

un enlazador flexible. Además de estos, se pueden utilizar los anticuerpos de dominio único (sdAbs) presentes en camélidos.[163]

1.4.1. Bibliotecas de anticuerpos

La construcción de bibliotecas de anticuerpos puede aprovechar diferentes fuentes de diversidad de genes. En principio, las bibliotecas pueden dividirse en bibliotecas inmunes y universales. Las bibliotecas inmunes suelen construirse a partir de tejidos o muestras de sangre de animales inmunizados[164], aunque también pueden generarse a partir de sangre procedente de seres humanos que hayan recibido una vacuna o de pacientes con una enfermedad[165]. Este tipo de bibliotecas están fuertemente sesgadas hacia un objetivo determinado, ya que el repertorio de anticuerpos ya ha sido preseleccionado, así como la afinidad optimizada *in vivo*. Para su construcción, se purifica la fracción de células mononucleares, donde se encuentran los linfocitos B, a partir de la sangre periférica. A partir del ARNm de estas células, se amplifican los genes de la región variable de las cadena pesada y ligera. Tras la unión de los fragmentos variables, los genes amplificados se clonan en un vector fagémido para generar así la correspondiente biblioteca de scFv en fagos filamentosos[166]. Cuando se trata de una biblioteca de anticuerpos monodominio, como es el caso de camélidos, se amplifican los genes de la región variable de la cadena pesada, tanto V_H como VHH.[167]

Desde el punto de vista práctico la construcción de bibliotecas de fragmentos VHHs es muy simple, especialmente si se compara con el trabajo tedioso que implica la construcción de bibliotecas de scFv o Fab. En este último caso, son necesarios varios juegos de cebadores. En humanos y animales es necesario una batería de cebadores por cada familia de genes V_H, rondas de PCRs, en las cuales se amplifican por separado las regiones variables de las cadenas pesadas y ligeras y luego se combinan por medio de una PCR overlap. Como resultado de este proceso se generan combinaciones V_L-V_H.[168]

Uno de los vectores fagémidos más utilizados para la construcción de bibliotecas de expresión de fagos es el pComb3x.[155] Este codifica una versión truncada de la proteína pIII que carece del dominio N-terminal vinculado al proceso de infección. Además, posee una secuencia líder que dirige el anticuerpo clonado en el extremo 5’ de la pIII truncada hacia el periplasma de la bacteria, donde hay un ambiente oxidativo que permite, por un lado, la formación de los puentes disulfuro y, por otro, el correcto plegamiento necesarios para la actividad del fragmento de anticuerpo. Asimismo, dicho vector presenta secuencias *tag* que facilitan la purificación por afinidad, como una cola de 6 histidinas.

Por el contrario, las bibliotecas universales pueden considerarse como "ventanillas únicas" para el aislamiento de anticuerpos específicos de antígeno contra prácticamente cualquier diana. Por lo tanto, este tipo de bibliotecas son de uso general. Las bibliotecas universales pueden subdividirse en naive, sintéticas y semisintéticas.[169] Las bibliotecas naive utilizan genes reordenados de la región variable procedentes de donantes no inmunizados y, en consecuencia, suelen acceder al repertorio de IgM.[170] Uno de los rasgos distintivos de estas bibliotecas es su naturaleza combinatoria, es decir, durante la construcción de la biblioteca se combinan aleatoriamente cadenas pesadas y ligeras.[171] Muchos antígenos terapéuticos son moléculas propias, pero durante la maduración de los linfocitos B se suelen eliminar los anticuerpos autorreactivos expresados en la superficie de los linfocitos B. Por lo general, se considera que, debido al ensamblaje combinatorio y aleatorio de las cadenas pesadas y ligeras, esta tolerancia inmunológica puede eludirse, al menos parcialmente, permitiendo la generación de anticuerpos dirigidos a autoantígenos. Las bibliotecas sintéticas pretenden maximizar la funcionalidad y para ello se diseñan y se sintetizan artificialmente los segmentos de la región variable. Para ello, se lleva a cabo una aleatorización de los CDRs, así como la introducción de modificaciones en la longitud del bucle y su composición de aminoácidos, maximizando el repertorio y funcionalidad de los anticuerpos resultantes.[169] En el caso de las bibliotecas semisintéticas se combina la diversidad de anticuerpos naturales y sintéticos, y se construyen a partir de secuencias de ADN naturales y sintéticas o partes de ellas.[172]

1.4.2. Selección de clones específicos

Una vez construidas las bibliotecas, las proteínas se seleccionan con las características deseadas, como la afinidad, la selectividad o la estabilidad, mediante un proceso denominado "panning". La metodología seguida en esta tesis está basada en los procedimientos establecidos por Barbas[168] y que, como se resume en la figura 18, consiste en incubar la biblioteca de fagos que expresan el fragmento de anticuerpo frente a la diana de interés. Para ello, se sensibiliza una superficie con el antígeno de interés. La inmovilización del antígeno puede realizarse en una superficie sólida, por ejemplo, en nitrocelulosa[173], perlas magnéticas[174], matrices de columnas[156], tubos de poliestireno[175], aunque normalmente se realiza en placas de microtitulación[155] con alta capacidad de unión a proteínas. Otras alternativas más complejas utilizan, antígenos biotinilados seguido de un "pulldown" empleando perlas recubiertas de estreptavidina[165] o líneas

celulares que expresen la diana elegida, lo que demuestra claramente la gran flexibilidad de la metodología Phage Display.

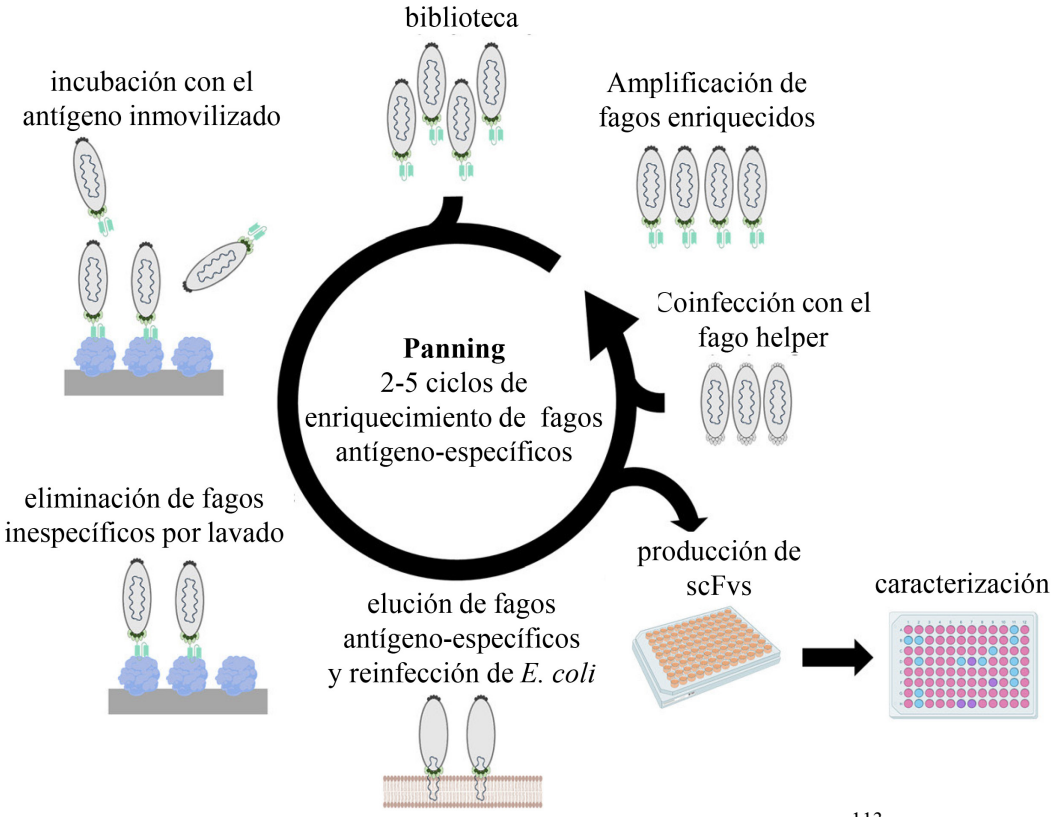

Figura 18: Esquema del proceso Phage Display.[113]

Seguidamente, se aplican pasos de lavado, donde se eliminan los fagos inespecíficos y, posteriormente, los fagos unidos al antígeno se eluyen enzimáticamente[176] o por cambio de pH[177] y se utilizan para la reinfección y la reamplificación en *E. coli*. Dado que las células de *E. coli* que albergan fagos sólo expresan la fusión anticuerpo: PIII se necesita de nuevo la infección del fago helper para la producción del resto de partículas del fago. Con dicho fin, se lleva a cabo la infección de *E. coli* con los fagos eluídos en la última ronda y se plaquea sobre LB-Agar para obtener colonias aisladas. Dichas colonias, donde cada una tiene en su interior un vector fagémido que codifica para un anticuerpo diferente, se crecen e induce la expresión del anticuerpo para analizar su reactividad por ELISA. Ya que el ADN que codifica para el anticuerpo seleccionado es encapsulado dentro de la partícula viral, se establece un vínculo físico entre el genotipo (ADN encapsulado dentro de la partícula viral) y el fenotipo (anticuerpo/proteína de fusión). Dicho vínculo representa una ventaja intrínseca de esta tecnología ya que es lo que nos permite la selección de clones individuales a partir de las millones de variantes presentes

en una biblioteca. Posteriormente, la información genética de los resultados positivos se recupera mediante secuenciación.[113]

1.4.3. Anticuerpos recombinantes obtenidos mediante Phage Display

1.4.3.1. Anticuerpos recombinantes como terapia

Actualmente existen más de 40 anticuerpos recombinantes terapéuticos y cientos se encuentran en fase de evaluación clínica pendiente de su aprobación. Este tipo de anticuerpos se utiliza como nuevo tratamiento en un amplio rango de patologías, como alergia, cáncer, autoinmunidad o enfermedades infecciosas.[128]

En este sentido, la tecnología de Phage Display de anticuerpos es una poderosa herramienta para seleccionar anticuerpos neutralizantes contra distintos marcadores biológicos propios de una patología, así como toxinas activas completas o ciertos dominios mediante el uso de bibliotecas de anticuerpos humanos con alta diversidad.[178–180] El objetivo de estos anticuerpos neutralizantes es, en la mayoría de los casos, bloquear la interacción de una determinada molécula con su diana celular, al bloquear el dominio de unión, o bloquear los dominios de translocación o enzimático si se trata de un toxina.[165,181] Algunos ejemplos son Raxibacumab (Abthrax) para tratar infecciones de *Bacillus anthracis*[182] y Foravirumab para el tratamiento la rabia[183].

En tratamiento de alergias, MEDI4212 (MedImmune) es un anticuerpo IgG1 anti-IgE que se generó utilizando Phage Display combinado con mutagénesis dirigida de las secuencias V_H y V_L para aumentar la afinidad por la IgE, haciendo que la de este sea 100 veces superior a la de Omalizumab.[184]

En terapia contra el cáncer, Adalimumab (Hamira) fue el primer anticuerpo obtenido mediante *Phage display* aprobado para su uso con humanos. A este anticuerpo le han seguido otros como Belimumab (Benlysta), que mejora la afinidad por su diana, el factor de necrosis tumoral (TNF). También se han desarrollado otros anticuerpos frente a otras dianas relacionadas con cáncer como Necitumumab (Portrazza), Ranibizumab (Lucentis) y Ramucirumab (Cyramza).[184]

Tabla 3: Anticuerpos recombinantes aprobados por la FDA para su uso en humanos.

Nombre	Tratamiento	Biblioteca	Formato de anticuerpo	Año de aprobación	Compañía	Ref
Adalimumab	Enfermedades inmunológicas e inflamatorias	Naive Fab	IgG1-κ	2002	AbbVie	[185]
Belimumab	Enfermedades inmunológicas	Naive scFv	Naive scFv	2011	GSK	[186]
Raxibacumab	Ántrax	Naive scFv	IgG1-λ	2012	GSK	[182]
Ramucirumab	Cáncer de pulmón	Naive Fab	IgG1-κ	2014	Eli Lilly	[187]
Necitumumab	Cáncer de pulmón	Naive Fab	IgG1-κ	2015	AstraZeneca	[188]
Ixekizumab	Psoriasis	Inmune Fab	IgG4-κ	2016	Eli Lilly	[189]
Avelumab	Cáncer de vejiga	Naive Fab	IgG1-λ	2017	Pfizer	[190]
Ranibizumab	Retinopatía diabética	--	IgG1-κ	2017	Roche	[191]
Moxetumomab	Leucemia	--	IgG1	2018	AstraZeneca	[192]
Caplacizumab	Cardiología	Inmune VHH	Nanoanticuerpo humanizado	2018	Sanofi	[193]
Lanadelumab	Angioedema hereditario	Naive Fab	IgG1-κ	2018	HGS	[194]
Emapalumab	Enfermedades inmunológicas	Naive scFv	Naive scFv	2018	NovImmune	[195]
Atezolizumab	Cáncer de pecho	Naive Fab	IgG1-κ	2019	Roche	[196]

1.4.3.2. Anticuerpos recombinantes para diagnóstico

Hasta la fecha, se han generado multitud de anticuerpos mediante Phage Display para aplicaciones de diagnóstico contra diferentes patógenos. Para tener resultados fiables en el diagnóstico es necesario anticuerpos con elevada afinidad y selectividad que permitan disminuir los límites de detección de los ensayos. Es por ello que la investigación en este campo parece estar en constante aumento, ya que el 44% de estos anticuerpos se han generado sólo en los últimos cinco años[128]. Se han producido anticuerpos frente a bacterias como *Mycobacterium tuberculosis*, *Salmonella Typhimurium* o *Listeria monocytogenes*[197–199]; así como frente a un amplia gama de patógenos eucariotas, por ejemplo, *Taenia solium*, protozoos, como *Cryptosporidium parvum*, *Plasmodium falciparum* o *Toxoplasma gondii* y hongos como *Aspergillus fumigatus*.[200–204] Además de los patógenos humanos, también se han obtenido anticuerpos recombinantes frente a patógenos veterinarios como *Myxobolus rotundus* (un patógeno de los peces) o *Babesia gibsoni* (un patógeno de los perros) y los patógenos de las plantas como *Aspergillus niger*, *Fusarium verticilloides* o *Sclerotinia sclerotiorum*.[205–209]

El uso de bibliotecas tanto universales como inmunes, ha permitido disponer de anticuerpos contra virus patógenos humanos como el virus del Dengue, el virus de la gripe, el VEEV, el norovirus, el coronavirus del SARS, el SARS-CoV-2 o la hepatitis C.[210–216] Otra fuente de anticuerpos monoclonales han sido las bibliotecas de genes de anticuerpos inmunes construidas a partir de huéspedes infectados, por ejemplo, Hantavirus, WEEV, el VIH, el SARS, el virus de la fiebre amarilla o el virus de la gripe.[217–222] Además, se han utilizado bibliotecas semisintéticas para generar anticuerpos contra, por ejemplo, el virus de la gripe.[223] Los virus de las plantas también se han abordado para la generación de anticuerpos, además de los virus humanos y animales.[224]

Por otro lado, los fagos no solo se pueden aplicar para el desarrollo de herramientas diagnósticas, sino que también se están proponiendo como alternativa a la antibioterapia. En 2013 se puso en marcha un estudio destinado a evaluar la fagoterapia en individuos con quemaduras infectadas por *E. coli* y *P. aeruginosa,* y en 2017 se inició un ensayo clínico para comparar las eficacias del tratamiento convencional junto a fagos frente a la terapia sin fagos en el tratamiento de las úlceras causadas por *S. aureus*. Esto abre una nueva ventana en la lucha contra la resistencia a antibióticos y tratamiento clínico.[225–227]

A pesar de la gran cantidad de anticuerpos recombinantes obtenidos mediante esta metodología para el diagnóstico de enfermedades e infecciones, hasta la fecha no se ha

recurrido a la metodología Phage Display para la mejora en el diagnóstico *in vitro* de alergias antibióticos BLCs.

De manera que, teniendo en cuenta el avance que ha supuesto el uso conjunto de Phage Display y la ingeniería de anticuerpos en la terapia y diagnóstico de enfermedades, es lógico considerar ampliar su uso con el fin de mejorar las prestaciones analíticas y clínicas de aquellos métodos diagnóstico poco eficaces, como es el caso de los métodos *in vitro* para la determinación de alergia a antibióticos BLCs. Esta metodología aporta las herramientas necesarias para la obtención de patrones de estandarización específicos que podrían utilizarse como control positivo o calibrador en estos métodos diagnóstico, lo que conduciría a una mejora en el diagnóstico y correcto etiquetado de los pacientes alérgicos, evitando los problemas asociados al uso de nuevos antibióticos.

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2. HIPÓTESIS

HIPÓTESIS

A diferencia de otras alergias, la hipersensibilidad a los antibióticos β-lactámicos está mediada por niveles muy bajos de sIgE. Sin embargo, los métodos *in vitro* utilizados para su diagnóstico no alcanzan los niveles de sensibilidad analítica requeridos, por lo que su sensibilidad clínica es muy baja (< 25%). Esta dificultad a la hora de discriminar correctamente entre pacientes alérgicos y no alérgicos ha conducido a que el 90% de la población etiquetada como alérgica realmente no lo sea y, por tanto, reciban antibióticos de nueva generación para el tratamiento de infecciones bacterianas comunes. Esto ha hecho que los planes de optimización del uso de antibióticos, implementados en el Plan nacional frente a la resistencia a antibióticos (PRAN), pongan el foco en la importancia que tiene un buen diagnóstico a la hora de etiquetar a un paciente como alérgico a algún antibiótico BLC e, incluso, en el desetiquetado de aquellos que no lo sean.

La baja capacidad diagnóstica de los métodos *in vitro* en uso hace necesario desarrollar herramientas analíticas que permitan detectar niveles más bajos de sIgE y así diagnosticar mejor los casos de alergia. Sin embargo, el desarrollo de nuevos métodos se ve frenado debido a la falta de materiales de referencia específicos de antibióticos BLC. Por lo tanto, es fundamental disponer de reactivos adecuados, para poder poner a punto procedimientos y estandarizar metodologías que permitan un diagnóstico más fiable con el que se puede abordar la identificación de verdaderos alérgicos.

La metodología Phage Display y la ingeniería de anticuerpos ofrecen las herramientas necesarias para la producción de proteínas recombinantes, específicas y de elevada afinidad, que mimeticen la función y la estructura de las sIgE. Esto permitiría alcanzar los retos que hasta ahora no se han podido superar en el diagnóstico *in vitro* de la alergia a antibióticos BLCs, es decir, la obtención de pruebas *in vitro* más sensibles y específicas.

Como ya se ha comentado, las proteínas recombinantes se caracterizan por producirse de manera sistemática, con buenos rendimientos y estar bien caracterizadas. Esto las convierte en una fuente ilimitada de material biológico para llevar a cabo estudios que permitan evaluar las prestaciones analíticas y diagnósticas de las diferentes pruebas. Su uso posibilitaría, además, la puesta a punto de ensayos con sistemas de cuantificación homóloga. Asimismo, al tratarse de inmunoglobulinas con una especificidad definida, permitiría realizar una estandarización de los métodos aportando información fiable y valiosa que garantizaría un buen diagnóstico y etiquetado.

3. OBJETIVOS

OBJETIVOS

De acuerdo con la hipótesis planteada, el objetivo principal de esta tesis es el diseño, obtención y caracterización de proteínas recombinantes con reactividad frente a antibióticos β-lactámicos.

Para ello, se establecen los siguientes objetivos parciales:

1. Mejorar la sensibilidad diagnóstica de las pruebas *in vitro* para la determinación de IgE específica en suero sanguíneo, mediante el estudio y puesta a punto de un inmunoensayo con detección quimioluminiscente.

2. Producir y caracterizar proteínas recombinantes que mimeticen la estructura de la IgE específica a antibióticos β-lactámicos.

3. Producir y caracterizar IgE específica recombinante para su uso como control positivo y patrón de estandarización de las pruebas *in vitro* de alergia a antibióticos β-lactámicos.

4. Evaluar el uso de proteínas recombinantes como calibradores específicos para la cuantificación de IgE específica a antibióticos β-lactámicos en un inmunoensayo siguiendo un esquema de calibración homóloga.

5. Establecer las prestaciones reales del ensayo y comparar los resultados obtenidos, utilizando un amplio conjunto de muestras representativas de suero de pacientes y controles previamente analizadas con los métodos de referencia.

4. RESULTADOS EXPERIMENTALES

4.1. CAPÍTULO 1

Determinación de sIgE a β-lactámicos mediante inmunoensayo quimioluminiscente

RESUMEN

Dentro de las pruebas *in vitro* para el diagnóstico de alergias, el inmunoensayo para la determinación de sIgE es la metodología más extendida. Sin embargo, los ensayos *in vitro* basados en la inmunodetección de sIgE a antibióticos BLCs se caracterizan por presentar baja sensibilidad analítica y clínica, lo que limita su uso a la hora de establecer un diagnóstico correcto.

Los inmunoensayos presentan gran variabilidad en cuanto a superficie y formato de ensayo, sin embargo, la placa ELISA se ha convertido en la plataforma por excelencia a la hora de llevarlos a cabo, ya que se puede encontrar prácticamente en cualquier laboratorio, permite analizar un elevado número de muestras al mismo tiempo y se llegan a alcanzar altos niveles de sensibilidad. Sin embargo, los grandes volúmenes de muestra requeridos y los largos tiempos de espera son dos de sus grandes inconvenientes a la hora de su utilización para el diagnóstico de alergias.

Es por ello por lo que el **capítulo 1**, en relación con el objetivo parcial 1, se centra en la puesta a punto de un inmunoensayo en placa ELISA con detección quimioluminiscente para la cuantificación de sIgE a antibióticos BLCs. Para ello se ha planteado la optimización del ensayo con el fin de aumentar la sensibilidad frente al de referencia (ImmnoCAP) a la vez que se disminuyen el volumen de muestra y el tiempo de ensayo.

Para alcanzar la elevada sensibilidad analítica requerida en este tipo de pruebas se incorpora la detección luminiscente, una alternativa a la detección colorimétrica con el potencial de aumentar la sensibilidad en varios órdenes de magnitud. Además, se utilizan nuevos determinantes antigénicos de penicilina G, penicilina V, amoxicilina y piperacilina conjugados a la proteína Histona H1, por lo que finalmente se consigue un ensayo quimioluminiscente multiparamétrico.

Dicho inmunoensayo se utilizó para evaluar el nivel de sIgE con éxito en una cohorte de 140 muestras de suero humano mostrando una buena sensibilidad y especificidad clínica, mejorando significativamente el carácter predictivo de las actuales pruebas *in vitro* de alergia a antibióticos BLCs, utilizando 25 µL de muestra.

4.1.1. Multiparametric Highly Sensitive Chemiluminescence Immunoassay for Quantification of β-Lactam-Specific Immunoglobulin E

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Multiparametric Highly Sensitive Chemiluminescence Immunoassay for Quantification of β-Lactam-Specific Immunoglobulin E

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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 the immunological determination of allergen-specific IgE, but the tests in the market lack the required sensitivity and specificity. In addition, 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 a carrier. The developed CLIA allowed the determination of BLC-specific IgE below 0.1 IU/mL, thus allowing identification of allergic patients with better sensitivity, using only 25 μL of a 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%), which significantly improve the predictive character of existing BLC-allergy *in vitro* tests.

INTRODUCTION

β-lactams (BLCs) antibiotics are the most widely used drug to fight bacterial infections worldwide due to their efficacy, safety, and cost, 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 with 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 cases.[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 that could directly determine the levels of drug-specific IgE (sensitization). A positive specific IgE response accompanied by a history of allergic symptoms makes 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 a 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 3 h, 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 BLCs 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 (radioallegrosorbent test), fluorescence enzyme assays (as ImmunoCAP), and ELISA (enzyme-linked immunosorbent assay).[11]

The 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 cutoff 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, and 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 indicates that even though approximately 15% of the world population are labeled as allergic to BLCs,[15] most diagnoses of BLC allergy are related to events that are not allergic in nature and, therefore, are associated with negative clinical and administrative outcomes, including the use of less desirable alternative antibiotics, longer hospitalizations, increasing antibiotic-resistant infections, and greater medical costs.[16] BLC allergy delabeling has become a global operationalizing focused on avoiding 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, and 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; therefore, it can represent a valid alternative to fluorescence enzyme-linked immunoassay (FEIA), being relatively simple and inexpensive for the assessment of serum sIgE for common allergens.[7] However, this method often requires large volumes of the 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, chemiluminescence immunoassay (CLIA). This technique has the potential to improve the sensitivity by at least 2−3 orders of magnitude compared to conventional colorimetric detection.[18] In this type of assays, luminol is used as a substrate for the HRP-labeled 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 purchased from Sigma-Aldrich (Madrid, Spain). Omalizumab was purchased from Novartis International AG (Basel, Switzerland). WHO reference IgE standard 11/234 was obtained from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom). Mouse monoclonal antibody anti-human IgE (α-IgE) was obtained from Eurofins Ingenasa S.A. (Madrid, Spain). Goat anti-mouse IgG preabsorbed (GAM-HRP) was purchased from Abcam (Cambridge, United Kingdom). The 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 was 10 mM sodium phosphate buffer, 150 mM NaCl, 0.05% Tween 20%, pH 7.4 (PBS-T).

High-binding white polystyrene ELISA plates were obtained from Costar Corporation (Cambridge, MA, USA), the ELISA plate washer was obtained from Nunc Maxisorp (Roskilde, Denmark), and the EnSpire Multimode Plate Reader was purchased 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, BLC 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 °C until use.

Assay Procedure for Multiparametric Chemiluminescence Immunoassay.

A scheme of the chemiluminescence immunoassay procedure is shown in Figure 1.1. First, white flat-bottomed polystyrene ELISA plates were coated with antigen solutions (3.0 mg/L) in a coating buffer (25 μL/well) for the direct determination of specific IgE to

β-lactam antibiotics. In addition, 25 µL of the 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, the 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 a 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 (1/2,000 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) was added to each well and incubated again for 15 min. After washing the wells as before, the peroxidase activity was measured by adding 25 µL of the enhanced chemiluminescent substrate solution previously diluted 1/10 in PBS. The luminescent signals were read at 450 nm using a multimode plate reader.

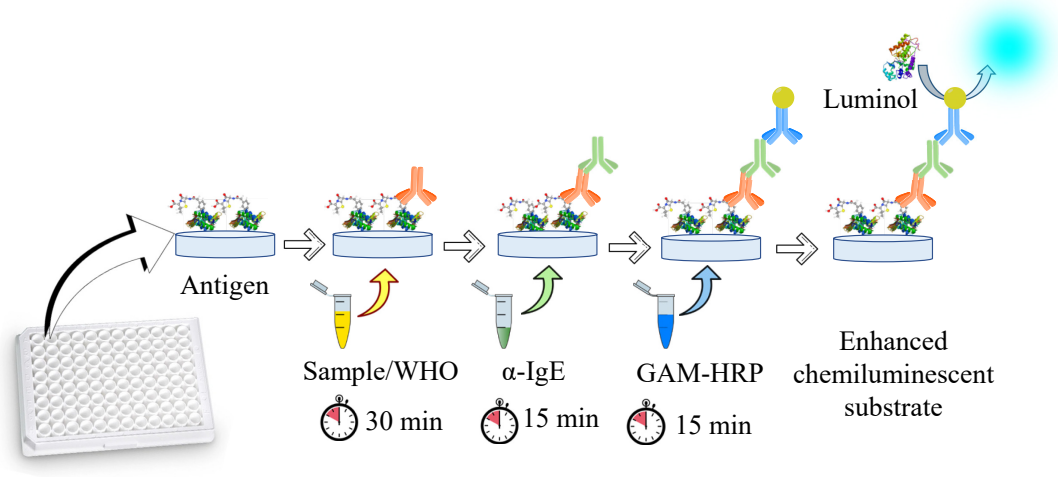

Figure 1.1: Scheme of the multiparametric chemiluminescence immunoassay in a 96-well microplate for the determination of specific and total immunoglobulin E (IgE) to β-lactam antibiotics in human serum.

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) were calculated by measuring the signal of the blank 10 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 a known specific IgE concentration, measured by the reference *in vitro* ImmunoCAP

method, was used as a 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) and incubated at room temperature for 60 min to induce clotting. After centrifugation at 2000 rpm for 15 min, the serum was aliquoted into cryovials and stored at −80 °C until use. A cohort of 71 allergic patients (allergic to at least one of the four BLC antibiotics under study) and 69 nonallergic to BLC individuals (controls) were included in the study. The clinical history of the patients included the result of the prick test to different BLC 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 a 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 assay 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 an 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 threefold serial dilutions (1/3−1/27) made up of control serum samples from nonallergic 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.

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

The volume of both the reagents and the sample and the total assay time were evaluated by carrying out four assays: 100 µL of the sample and each reagent and a total assay time of 3 h (standard protocol), and 50, 25, and 10 µL of sample and each reagent with a total assay time of 1 hr. Once the test was performed, as can be seen in Figure 1.2, a proportional reduction in the luminescence signal was observed when the test volumes and time decreased.

By analyzing each of the experiments separately, the data statistics show that when 10 µL of the 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.

Therefore, taking into account all the results, the test was switched to use a volume of 25 µL and 1 h 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] and 40 µL used in the most up-to-date ImmunoCAP (Phadia) equipment.[22]

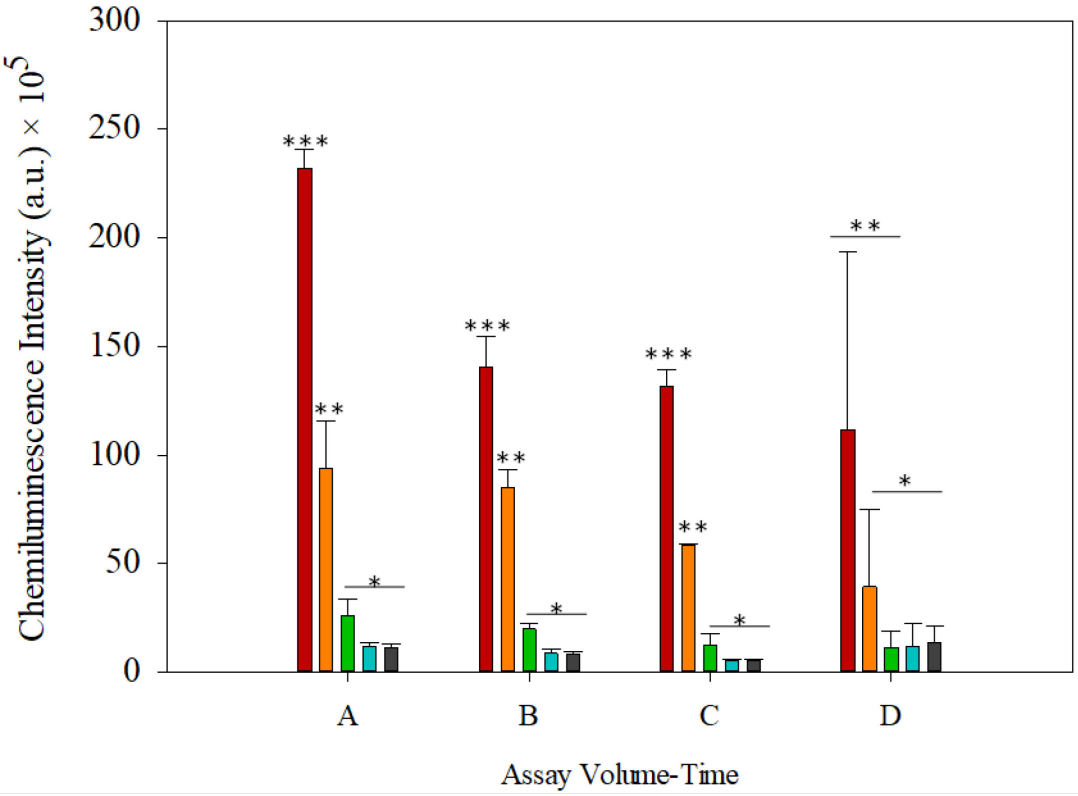

Figure 1.2: Results of the optimization assays (A−D) with Histone-AMX as an antigen and a representative positive serum sample. A: Standard protocol (100 µL of volume used and total assay time 3 h). B, C, and D: 50, 25, and 10 µL of volume used, respectively, and total assay time was approximately 1 h. The group of bars (A−D) corresponds to specific IgE concentration to amoxicillin [IgE]. From the left to the right, the concentrations were 3.3, 1.1, 0.35, 0.12, and 0 IU/mL.

Evaluation of Antigen Performances.

Antigens are the essential elements 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 BLC 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 for activating the immune system in order to produce specific IgE antibodies against a particular epitope. In this study, histone H1 was used as a carrier molecule to conjugate the BLC antibiotics. Histone H1 is a lysine-rich protein

that contains tens of primary amines available for coupling BLC 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 a known concentration of specific IgE for amoxicillin (7.0 IU/ mL as measured by ImmunoCAP), using threefold serial dilutions (1/3−1/81). Dilutions were made with a pooled (n = 69) control serum sample as the diluent. As is shown in Figure 1.3, the linearity was good over a wide range of dilutions, 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 0.09 IU/mL.

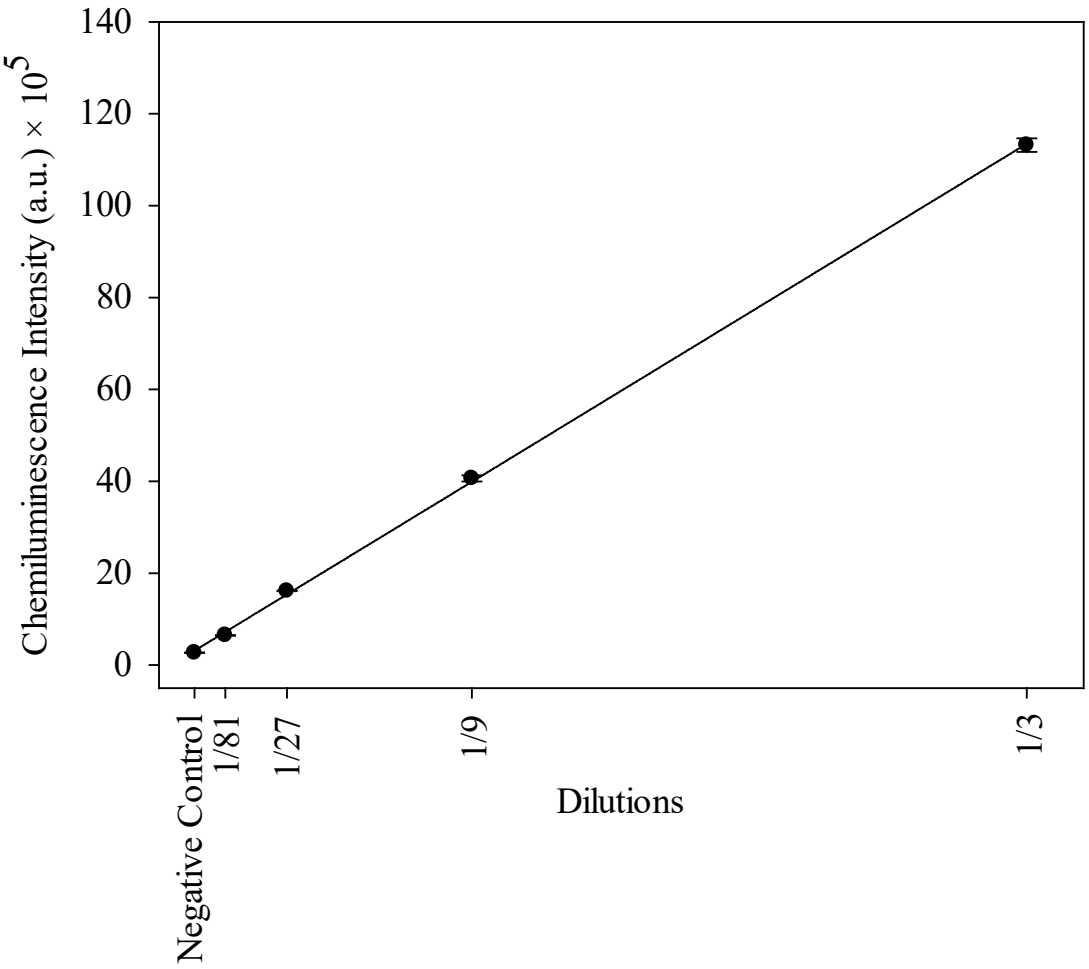

Figure 1.3: Dilution linearity study ($r^2 = 1$) of the assay using a serum sample as a matrix to evaluate the accuracy of the CLIA.

The rest of the histone-derived antigens (penicillin G, penicillin V, and piperacillin) performed well, showing an LOD of 0.03 IU/mL for penicillin G and penicillin V,

calculated from the results obtained with the linearity-of-dilution experiments (Figure S1.3).

Calibration Method.

A reliable quantitative method must report the results in units traceable to an international standard. Nowadays, it is not possible to use an individual-specific IgE for BLC antibiotics to perform a homologous calibration method. Therefore, the calibration method mostly accepted is a heterologous interpolation of specific IgEs from a single total immunoglobulin reference curve. As it can be observed in Figure 1.4 (solid line), the signal (n = 10) fits well to a four-parameter logistic curve, achieving an LOD of 0.04 IU/mL and dynamic response ranging from 0.5 to 8.0 IU/mL, following a point-to-point calculation method approach. On the assessment of the precision of the CLIA, CVs ranged from 1.6 to 8.6% and resulted in a linear regression equation with a correlation coefficient (r) of 0.999. Likewise, a representative sample with a 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 1.4 (dashed line), the signal (n = 6) fits well to a four-parameter logistic curve, achieving an LOD of 0.03 IU/mL. In the homologous curve, CVs ranged from 0.5 to 2% and resulted in a linear regression equation with a correlation coefficient (r) of 1.

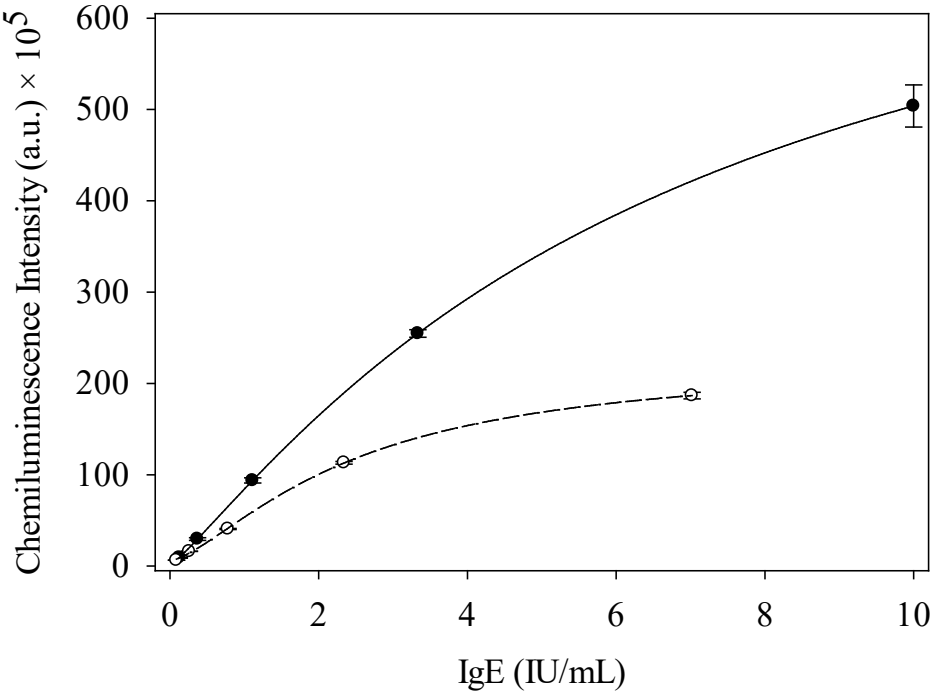

Figure 1.4: Heterologous (solid line) and homologous (dashed line) calibration curves for specific IgE to amoxicillin by CLIA.

Spike-and-recovery tests were carried out to evaluate the differences in assay response, using representative serum samples. The results are shown in Table 1.1. As it can be seen, the recovery values ranged between 65 and 121%, revealing that the components in the sample matrix could cause significant differences in assay response. The relative standard deviation values were below 10% for all dilutions.

Table 1.1: Recovery results obtained for moderate-level BLC-specific IgE human serum samples by CLIA

Sample	[a]DF	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 Performance of the CLIA.

A cohort of 140 sera collected from patients allergic to one or more BLC antibiotics (71) and nonallergic to BLC antibiotics (69) were analyzed, 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; the result of the 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 nonallergic individuals. Results that were above the 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; these figures are below the current internationally accepted cutoff concentration for allergy diagnostics (0.35 IU/mL). For CLIA, the cutoff was 0.04 IU/mL, providing a clinical sensitivity of 64.6% and a clinical specificity of 100%.

After the analysis of the controls (nonallergic patients), it is observed that both methods identify these samples as negative, since the results obtained are below the LOD of each method. The absence of false positives ratifies the good performance of the CLIA method. Table 1.2 lists the number of patients (allergic) analyzed, showing the amount of positive (> LOD) and negative (< LOD) results obtained by both methods.

Table 1.2: 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 can detect a greater number of sensitive people when analyzing samples from allergic patients, using the heterologous calibration (Table 1.3). In addition, the results for positive tests were compared between the two methods. Despite being analytical systems that use different antigenic determinants, the scatter diagram (Figure 1.5A) shows a good correlation (0.8670, $p < 0.0001$) between ImmunoCAP and CLIA, revealing a good precision of the proposed methodology. Though there are slight differences in measuring the concentration of specific IgE, CLIA performs an underquantification 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; underquantification 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; however, this behavior changes at higher concentrations: lower values are obtained than that with ImmunoCAP but higher than that when using the WHO curve. This difference when using the curves (Figure 1.4) 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 underquantification when using the WHO curve.

In order to compare CLIA with the reference method at the diagnostic level, we exclusively relied 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 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 cutoff of 0.35 IU/ mL for amoxicillin and uses a poly-L-lysine-based antigen. All these reasons might probably be the cause of the poor correlation observed at low concentrations. Receiver operating characteristic (ROC; Figure 1.5B) analysis showed a good area under the curve (AUC) for CLIA. Indeed, both *in vitro* tests showed high diagnostic specificity since no false positives were detected. Regarding diagnostic sensitivity, 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 the reference test. Indeed, a threefold increase in sensitivity was achieved, which confirms the strength point of the developed CLIA method.

Analysis of the results showed a high percentage of cross- reactivity that exists in BLC allergy. Of the 17 patients detected as allergic to penicillin G, none were 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 BLC 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 antibiotic 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 BLC ring in their

molecular structure and are distinguished from each other by different side chains. Sometimes, the 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 an IgE capable of recognizing it. This information provides us a predictive diagnosis, which will help the patient avoid contacting with an antibiotic that can trigger a high-risk allergic reaction.

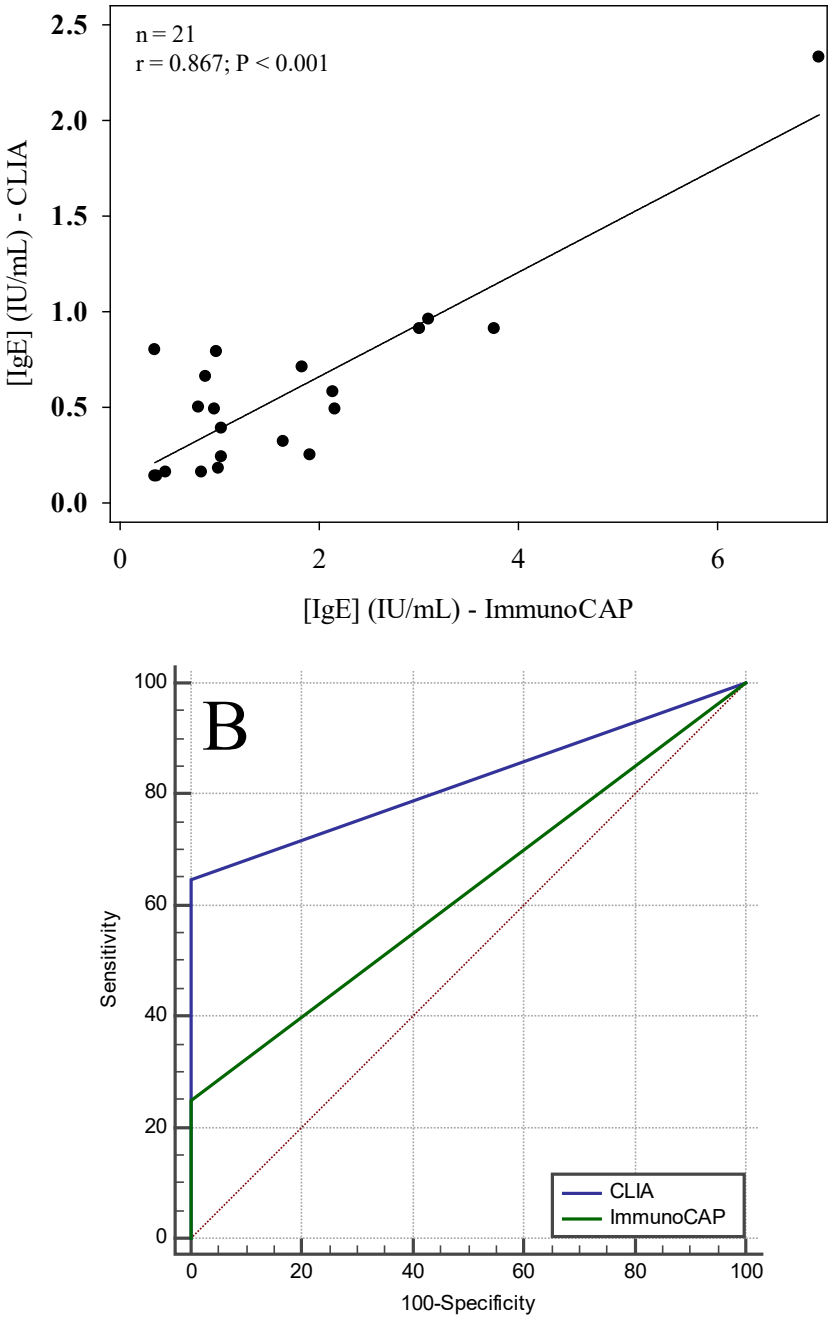

Figure 1.5: [A] Scatter diagram and regression line of intermethod comparison between CLIA and ImmunoCAP. [B] ROC analysis representing the AUC. Sensitivity and specificity of CLIA as compared to ImmunoCAP for amoxicillin (n = 140 values).

Table 1.3: 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

	Penicillin G		Amoxicillin			Penicillin V		Piperacillin
Patient	**CLIA[a]**	**ICAP**	**CLIA[a]**	**CLIA[b]**	**ICAP**	**CLIA[a]**	**ICAP**	**CLIA[a]**
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

	Penicillin G		Amoxicillin			Penicillin V		Piperacillin
Patient	**CLIA[a]**	**ICAP**	**CLIA[a]**	**CLIA[b]**	**ICAP**	**CLIA[a]**	**ICAP**	**CLIA[a]**
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

	Penicillin G		Amoxicillin			Penicillin V		Piperacillin
Patient	**CLIA[a]**	**ICAP**	**CLIA[a]**	**CLIA[b]**	**ICAP**	**CLIA[a]**	**ICAP**	**CLIA[a]**
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

CONCLUSIONS

The optimization of a multiparametric luminescence detection immunoassay developed in this study makes it possible to determine a 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 the multianalyte CLIA for screening antibiotic allergies. The use of a 96-well plate allows the analysis of a specific IgE against four BLC antibiotics from seven patients simultaneously and obtaining results in only 1 h. 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 64.6% 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 a lower detection limit to those of ImmunoCAP but render 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 labeled as allergic to BLCs when they are not. This incorrect labeling is associated with negative clinical and administrative outcomes, including the use of less desirable alternative antibiotics, longer hospitalizations, increasing antibiotic-resistant infections, and greater medical costs. CLIA makes it possible to increase the clinical sensitivity while maintaining 100% clinical specificity, which translates into no false positives, contributing to “delabeling”, one of the main challenges in allergy diagnosis today.

On the other hand, antibiotics are the main cause of immediate perioperative hypersensitivity (POH) reactions in countries such as Spain, the United States, and the United Kingdom, where they represent 44−59% of IgE-mediated POH.[23] The most frequently implicated antibiotics are BLCs, especially amoxicillin because of its widespread use. Due to the delicate state of the patients after an operation, the use of CLIA would allow a safe identification of these antibiotics that cause the allergic reaction.

In summary, we have developed a multiparametric immunoassay with luminescence detection that can be performed in any clinical laboratory, which allows the determination of specific IgE below 0.1 IU/mL with only 25 μL of serum and 1 h 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 an inexpensive, fast, and simple way.

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 to the final version of the manuscript.

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SUPPORTING INFORMATION

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

1. Blocking step study.

The long experience of the research group in the use of serum samples has shown that the high content of proteins present in human serum acts as a blocker of non-specific absorption. In order to verify our hypothesis, a study was carried out in which the assay was compared with and without a blocking step (Figure S1.1). The statistical analysis of the data through multiple regression analysis using SPSS Statistics (IBM) showed that there are no statistically significant differencesbetween both methodologies (p=0.265).

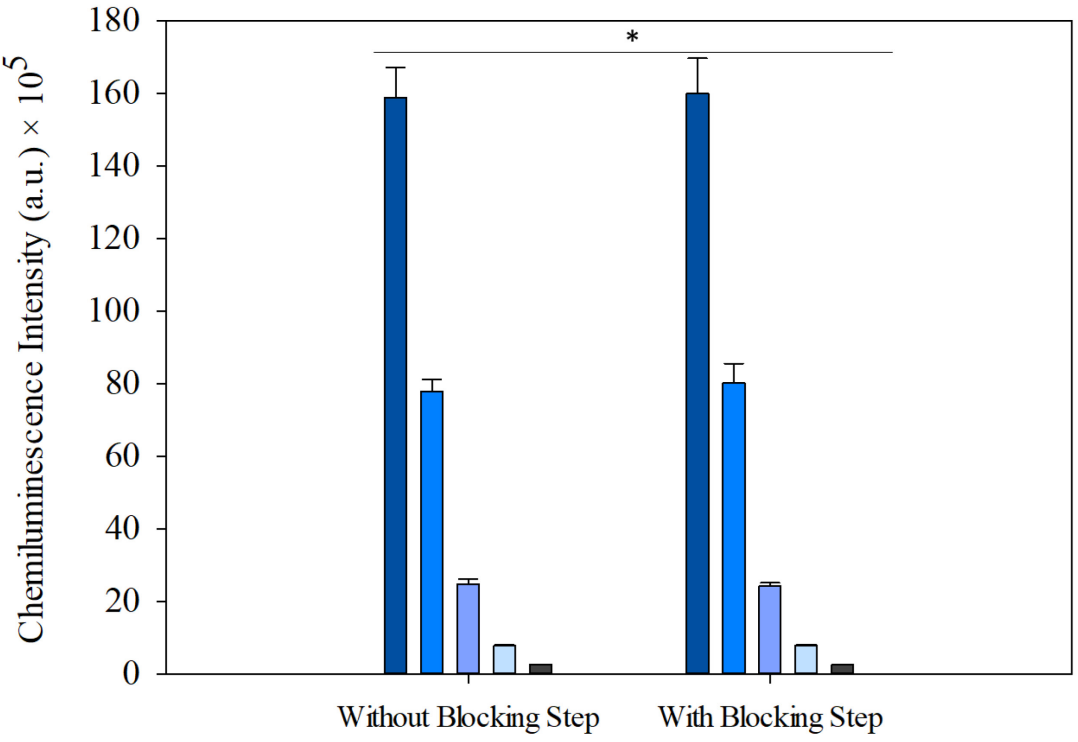

Figure S1.1: Results of the study of the use of the blocking step in CLIA. The group of bars correspond to specific IgE concentration to amoxicillin, from the left to the right the concentration was 5.0, 1.0, 0.50, 0.10 and 0 IU/mL.

2. Coating amount of H1-BLcs antigen study

In the development of this assay, a titration study was carried out to determine the amount of coating antigen with better analytical features.

As can be seen in figure S1.2A, an increase in the concentration of coating antigen causes a signal increase. Concentrations lower than 3 mg/L do not allow detecting low concentrations of sIgE (0.1 IU/mL). However, increasing the coating antigen concentration resulted in an increase in background noise (Figure S1.2B). Therefore, as mentioned in the manuscript, 3 mg/L was the optimal coating antigen concentration.

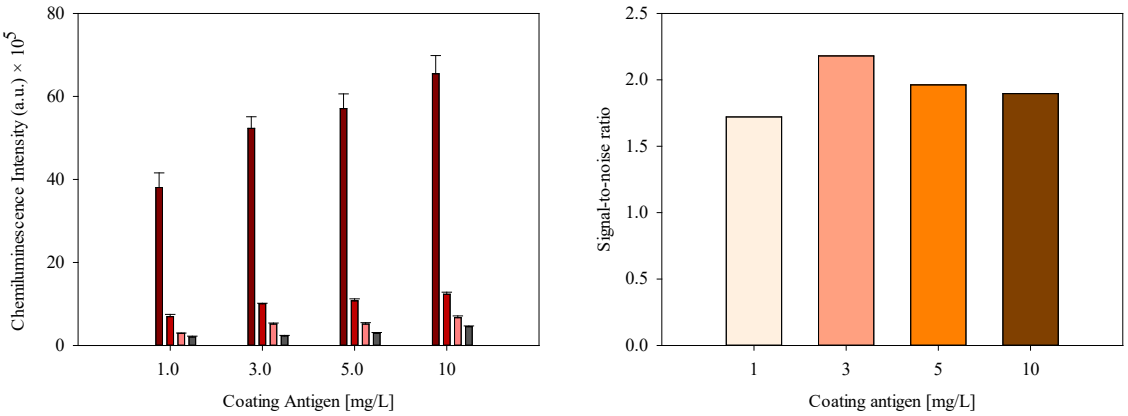

Figure S1.2: [A] Results of the titration study for coating antigen. The group of bars correspond to specific IgE concentration to amoxicilin, from the left to the right the concentration was 1.0, 0.50, 0.10 and 0 IU/mL. [B] Result of the signal-to-noise ratio study.

3. Sample Dilution study

In this study, the H1-AMX antigen was chosen as the model antigen, however, the other antigens have similar analytical characteristics. In the case of H1-AMX, the linearity dilution study is described in the manuscript. For Penicillin G (Figure S1.3A) and Penicillin V (Figure S1.3B), the same study was performed. A sample with known concentration of sIgE (3.0 and 3.7 IU/mL, respectively) was chosen and a pooled negative control human serum sample (n=69) was used as diluent. In both cases, the dilution 1/27 was significantly different from the negative control used as blank. The calculated limit of detection was 0.03 IU/mL.In the development of this assay, a titration study was carried out to determine the amount of coating antigen with better analytical features.

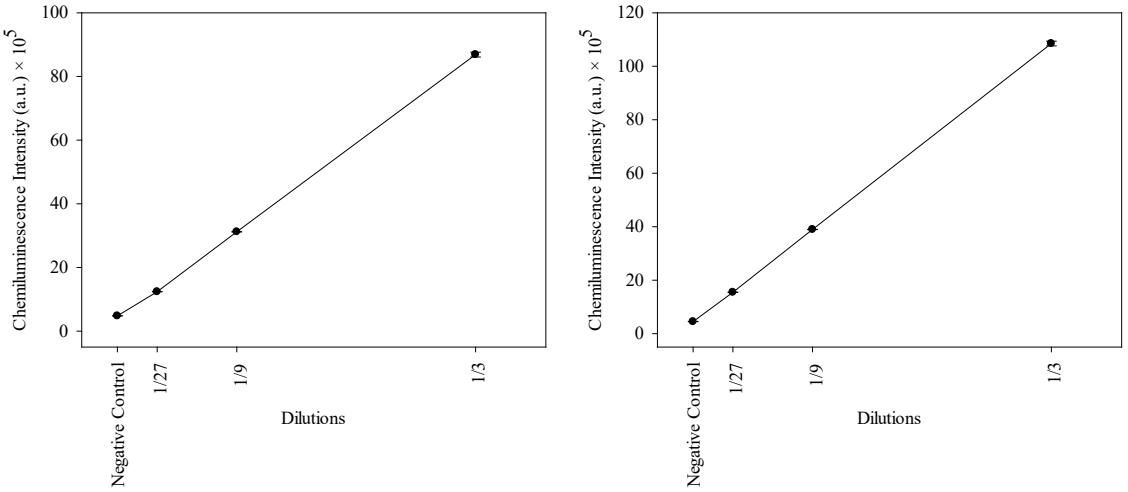

Figure S1.3: Dilution linearity study using a reference serum sample to evaluate the analytical performances of the CLIA for H1-PG (A) and H1-PV (B).

4. Interactive dot diagram.

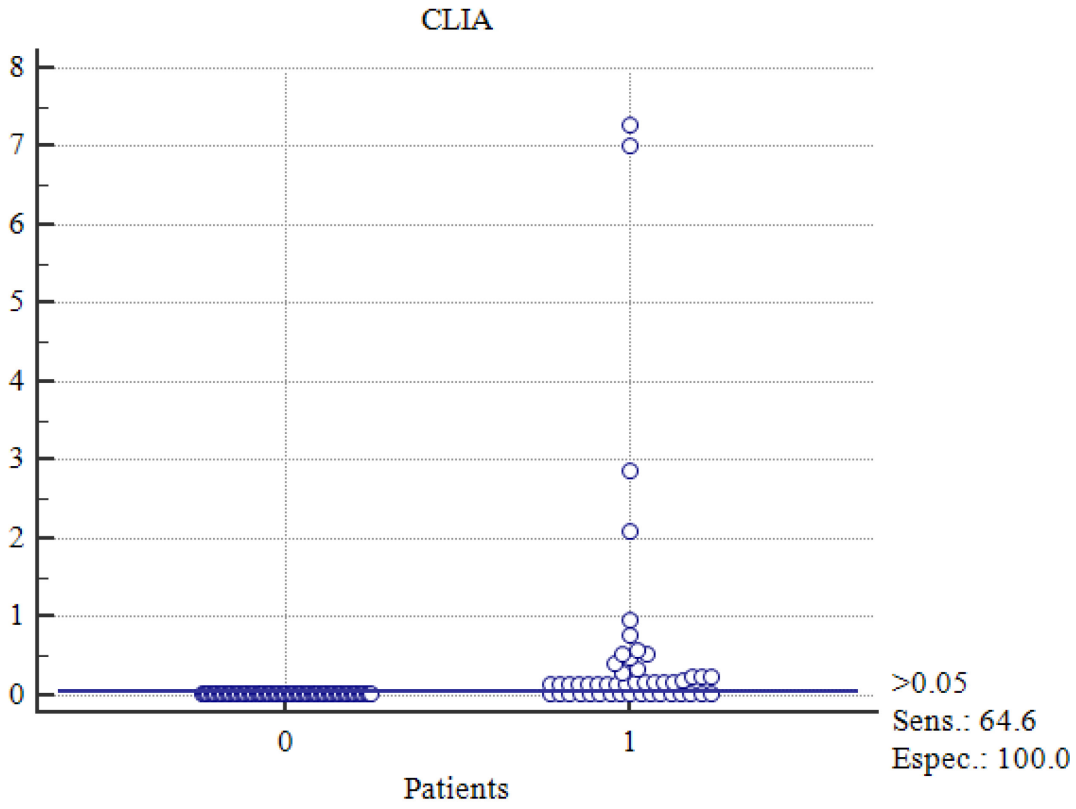

Figure S1.4: Interactive dot diagram. A cutoff of 0.05 IU/mL provides a clinical sensitivity of 64.6 % and a specificity of 100% for the cohort of tested samples.

4.2. CAPÍTULO 2

Producción de nanoanticuerpos miméticos de sIgE

RESUMEN

En el **capítulo 1** se ha descrito la puesta a punto de un inmunoensayo multiparamétrico en placa ELISA que permite determinar niveles de sIgE a antibióticos BLCs en suero por debajo de 0.1 IU/mL. Esta disminución del límite de detección con respecto al método de referencia va acompañada de un aumento de la sensibilidad clínica (64%) manteniendo una especificidad del 100%. Sin embargo, se ha puesto de manifiesto que la calibración heteróloga proporciona resultados más dispares que la calibración homóloga al comparar los resultados del CLIA con ImmunoCAP.

Es por ello, que en el **capítulo 2** se describe la producción de proteínas recombinantes con características similares a las sIgE de antibióticos BLCs. Para ello se recurre a un vector de expresión pINQ-bis para producir de modo recombinante proteínas formadas por dos anticuerpos de dominio único (nanoanticuerpos) unidos a través de un enlazador peptídico corto. De esta manera se obtiene una construcción recombinante en la que uno de los nanoanticuerpos reconoce selectivamente al determinante antigénico de un antibiótico BLC, mientras que el otro se une específicamente al parátopo de un anticuerpo detector anti-IgE (Omalizumab). De esta manera se obtienen proteínas recombinantes muy estables que pueden producirse indefinidamente a partir de su secuencia.

Concretamente, en la primera parte de este capítulo (**sección 4.2.1**), en relación con el objetivo parcial 2, se describe el diseño, producción y caracterización de dicha construcción biespecífica. Los resultados obtenidos son producto del trabajo llevado a cabo durante la estancia realizada en el laboratorio del Dr. Gualberto González Sapienza. Así, utilizando la metodología Phage Display y técnicas de biología molecular, se generaron proteínas específicas frente a aztreonam y amoxicilina, capaces de unirse por un domino al determinante antigénico correspondiente y ser reconocidas, a su vez, por Omalizumab. Como resultado, se obtuvieron proteínas estables, específicas y totalmente caracterizadas que podrían utilizarse como reactivos en el diagnóstico *in vitro* de alergias.

4.2.1. Bispecific single-domain antibodies as highly standardized synthetic calibrators for immunodiagnosis

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Bispecific Single-Domain Antibodies as Highly Standardized Synthetic Calibrators for Immunodiagnosis

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ABSTRACT

Commonly, serological immunoassays and diagnostic kits include reference standard reagents (calibrators) that contain specific antibodies to be measured, which are used for the quantification of unknown antibodies present in the sample. However, in some cases, such as the diagnosis of allergies or autoimmune diseases, it is often difficult to have sufficient quantities of these reference standards, and there are limitations to their lot-to-lot reproducibility and standardization over time. To overcome this difficulty, this study introduces the use of surrogate recombinant calibrators formulated on the basis of two single-domain antibodies (nanobodies) combined through a short peptide linker to produce a recombinant bispecific construct. One of the nanobodies binds to the cognate analyte of the target antibody and the second is specific for the paratope of the secondary detecting antibody. The bispecific nanobody inherits the outstanding properties of stability and low-cost production by bacterial fermentation of the parent nanobodies, and once calibrated against the biological reference standard, it can be reproduced indefinitely from its sequence in a highly standardized manner. As a proof of concept, we present the generation and characterization of two bispecific calibrators with potential application for the diagnosis of allergy against the antibiotics aztreonam and amoxicillin in humans.

INTRODUCTION

Standards are essential materials for analysis and primarily used as a reference for the quantification of analytes in samples. Many diagnostic tests use standards-based biological preparations (e.g., serum), which, due to their complexity, cannot be fully characterized by physicochemical methods. These preparations are commonly assigned a value of arbitrary units (AU), which are then used to express the test results. To ensure an adequate level of harmonization, organizations such as the World Health Organization

sponsor international multicenter studies to validate the use of biological reference materials and assign them a value in international units (IU).[1] For antibody detection tests, these standards are selected sera of human or animal origin, and diagnostic test developers use them as a calibration reference to transfer their value to a new serum masterlot, which then constitutes the standard reagent of the new test.[2] However, due to their biological origin, the reference sera are finite and their substitution can give rise to reproducibility problems due to batch-to-batch variations. Furthermore, in some diagnostic situations, the reference serum does not exist or it is difficult to ensure sufficient supply for its use as an assay reagent, particularly for isotypes other than IgG, such as the case of obtaining IgM-reactive sera to an infectious disease in the context of a population with a high degree of vaccination or the limited availability of IgE for rare allergies. In these situations, an interesting alternative to advance in the standardization of the test results is the use of well-defined and easily reproducible reagents as surrogate standards (calibrators).

In diagnostic assays to monitor levels of specific antibodies, the replacement of the seropositive reference serum or plasma requires design of a reagent that mimics the antigen-recognition ability of the specific antibodies being measured while providing binding sites for the secondary antibody used for detection. The strategy commonly used for this purpose has been to combine the human Fc region of interest with the antigen-binding site of heterologous antibodies obtained by hyperimmunization with the test antigen. Initially, this approach was achieved by chemical conjugation of whole antibodies,[3] but later these calibrators were produced as recombinant chimeric antibodies by joining the targeted human Fc region with Fab or scFv fragments specific for the antigen of interest.[4–6] The Fc region can also be combined with Fc receptors (FcγR) to build $(FcγR)_2Fc$ chimeras that can be loaded with hyperimmune polyclonal antibodies to the target antigen.[7] While providing a solution to the problem of standardization, these constructs need to be expressed in eukaryotic systems and are laborious and expensive to produce. In addition, the scFv components often present difficulties associated with their low stability and high propensity to aggregate,[8] which may require additional stabilization steps through optimization of the linkers, framework region, introduction of interchain disulfide bonds, etc.[9,10]

In this study, we introduce the use of single-domain antibodies (nanobodies) as modular elements to produce highly standardized recombinant calibrators or positive controls for immunoassays. These single-domain antibodies are derived from the variable

domain (VHH) of heavy chain antibodies (HcAb) found in camelids. These molecules have key advantages over conventional antibody fragments.[11] Thus, due to their monodomain nature, the loss of activity produced by heavy and light chain shuffling does not occur as in the case of conventional antibodies, so their specificity is preserved intact during the selection process by phage display as well as during their recombinant expression.[12] In addition to being raised in a simpler way, nanobodies have superior physicochemical properties than conventional antibody fragments in terms of solubility and stability and are easily produced by fermentation using *Escherichia coli* (*E. coli*).[13,14] A salient property of nanobodies is their ability to act as building blocks that can be fused in tandem to form bispecific antibodies. In this way, recombinant calibrators can be generated by combining a nanobody against the antigen of interest with a nanobody against the antigen-binding site of the secondary antibody. The production of this type of recombinant calibrators is facilitated by the fact that it is fairly easy to generate anti-idiotype nanobodies because the paratope of the monoclonal antibody used for immunization turns out to be the most immunogenic region for the immunized animal.[15,16]

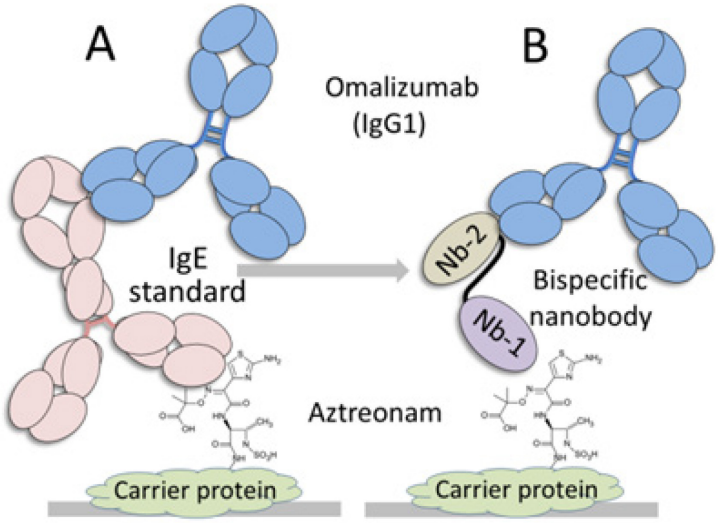

Figure 2.1: Schematic representation of the use of bispecific nanobodies as surrogate calibrators for biological standards. In the case of the immunodiagnosis of allergy to antibiotics, aztreonam in this example, the standard of IgE from patient sera is replaced by a bispecific nanobody composed of a nanobody that reacts with the adduct of the antibiotic, which is fused through a linker to a second nanobody that reacts with the paratope of the anti-IgE detection antibody (Omalizumab).

As a proof of concept, in this study, we developed recombinant calibrators for an allergy test for the antibiotics aztreonam and amoxicillin as outlined in Figure 2.1. The chosen models represent typical examples in which, as it occurs for other allergies against

antibiotics, the absence of reference sera makes it difficult to compare the results obtained in different laboratories.

MATERIALS AND METHODS

Materials.

Bovine serum albumin (BSA), D-biotin, isopropyl β-D-1-thiogalactopyranoside (IPTG), LB Broth (Miller), 3,3′,5,5′-tetramethylbenzidine (TMB), Tris base, Tween20, and other chemicals were purchased from Sigma (St. Louis, MO, USA). The anti-hemagglutinin epitope (anti-HA) antibody conjugated to horseradish peroxidase (HRP) was purchased from Sigma (Cat No. 12013819001), the anti-histidine (anti-His) antibody conjugated to HRP (Cat No. ab1187) and anti-human IgG antibody−HRP (Cat No. ab97225) were purchased from Abcam (Cambridge, UK), and streptavidin−HRP was purchased from Pierce (Rockford, IL, USA). Molecular biology reagents and *E. coli* One Shot BL21 (DE3) cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA). *E. coli* ER2738 electrocompetent cells were obtained from Lucigen Corporation (Middleton, WI, USA). The Omalizumab antibody was obtained from Novartis International AG (Basel, Switzerland). An HRP conjugation kit (Cat No. ab102890) from Abcam (Cambridge, UK) was used to prepare HRP-conjugated Omalizumab. The stocks of each Ig class and subclass (Cat Nos. 090701, 090704-M, 090704-1M, 090705, 090707-2M, 090707-4M, and 090713) were obtained from Athens Research & Technology, Inc. (Athens, GA, USA). Chromatography columns were obtained from GE Healthcare (Piscataway, NJ, USA). For the design of genes and primers, SnapGene software was used (from Insightful Science; available at snapgene.com). Primers and genes were obtained from General Biosystems Inc. (Morrisville, NC, USA). The β-lactam antibiotics were conjugated to keyhole limpet hemocyanin (KLH) or human serum albumin (HSA) as described by Quintero-Campos et al.[17]

Library construction.

A 3-year-old llama (*Lama glama*) from Lecocq Municipal Park Zoo (Montevideo) was immunized with four doses of 900 μg of purified Omalizumab and 500 μg of aztreonam and amoxicillin antibiotics conjugated to the HSA protein in incomplete Freund's adjuvant by subcutaneous injection. Ten days after the third booster, 200 mL of blood was drawn. Peripheral blood mononuclear cells were isolated using Histopaque-

1077 gradients (Sigma, St.Louis, MO, USA) according to manufacturer's instructions, and the total RNA from 10^8 cells was extracted using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), which was quantified spectrophotometrically and reverse-transcribed using oligo dT and RevertAID reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Then the genes of the heavy chain variable domains of the conventional (VH) and heavy chain only antibody (VHH) isotypes were amplified by polymerase chain reaction (PCR) as previously described.[18] The VH/VHH gene products were *Sfi*I-digested, cloned in the phagemid vector pComb3X (from Dr. Barbas, The Scripps Research Institute, La Jolla, USA), and electroporated into competent *E. coli* ER2738 cells. The transformed cells were cultured and superinfected with helper phage M13KO7 (New England Biolabs, Ipswich, MA, USA) at the beginning of the exponential phase to generate the phage library.[18]

Panning for the selection of anti-Omalizumab and anti-antibiotic nanobodies.

Panning for the selection of anti-Omalizumab nanobodies involved a first step of preadsorption to remove reactive clones against epitopes other than the paratope of Omalizumab. Preadsorption was performed by incubating $1 \cdot 10^{11}$ colony-forming units of the VH/VHH library on high-binding 8-well strips (Greiner Bio-One, Monroe, NC) coated with 1 μg/well human IgG1 for 1 h at room temperature (RT). Phage supernatants were recovered and added to four wells coated with 1 μg/well Omalizumab and incubated for 2 h at RT. The wells were washed 10 times with phosphate buffered saline (PBS)−0.05% Tween 20, followed by an incubation for 30 min at RT with PBS−0.05% Tween 20 and washed again 10 times. The elution of bound phages was carried out by adding 50 μL/well of 10 mg/mL trypsin and incubating for 30 min at 37 °C with agitation. Finally, the phage output was titrated and used for subsequent amplification in *E. coli* ER2738 for a second round of selection.

Panning for selection of nanobodies against β-lactam antibiotic determinants did not require a preadsorption step and was carried out as described above in well strips coated with 0.5 μg/well antibiotic conjugated to the KLH protein.

High-throughput expression of nanobodies.

DNA from the final/second output of the panning of Omalizumab was amplified by infecting 0.5 mL of cell culture (OD600nm = 1.0) with 50 μL of output phage. The infected cells were diluted in a flask containing 9.5 mL of SB broth with 20 μg/mL ampicillin and cultured overnight at 37 °C. Plasmid DNA was purified using a GeneJet

miniprep kit (Thermo Fisher Scientific, Waltham, MA, USA), *Sfi*I-digested, and cloned into the pINQ-BtH6 vector. The ligation product was electroporated into competent *E. coli* BL21 (DE3) cells carrying the pCY216 vector for overexpression of the biotin ligase of *E. coli*, and parallel cultures of individual colonies were then produced as described before.[19] Briefly, 89 colonies were inoculated to 500 µL of LB medium containing 50 µg/mL kanamycin, 35 µg/mL chloramphenicol, 0.04% of L-arabinose, and 100 µM D-biotin in 96-deep-well culture blocks (Greiner Bio-One, Monroe, NC). The expression of biotinylated nanobodies was induced at OD600nm = 0.6 with 10 µM IPTG overnight at 28 °C with shaking. The bacterial pellets were harvested by centrifugation, resuspended in PBS, lysed by four freeze−thaw cycles followed by 30 min of sonication in a sonicator bath, and incubated for 2 h at 37 °C to promote efficient biotinylation. The cell lysates were centrifuged and the supernatants were collected to test their reactivity. For this, each supernatant was analyzed in enzyme-linked immunosorbent assay (ELISA) plates coated with 0.2 µg/well Omalizumab, human IgG1, and human IgG, and the bound nanobodies were then detected with a streptavidin−HRP conjugate (Pierce, Rockford, IL).

The screening of anti-antibiotic determinant nanobodies was performed by directly picking isolated colonies from the second round of panning. In this case, individual colonies were cultured/grown in SB broth−ampicillin, and nanobody expression was induced with 1 mM IPTG overnight at 37 °C. After centrifugation, the supernatants were collected and assayed in ELISA plates coated with 0.2 µg/well KLH-conjugated antibiotic aztreonam, meropenem, ceftriaxone, amoxicillin, or penicillin G. Finally, bound nanobodies were detected with an anti-HA−HRP conjugate.

Construction of the pINQ-bis vector.

The pINQ-bis 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 an ompA signal peptide, the anti-β-lactam Nb flanked by two *Sfi*I restriction sites, a spacer of GQAGR(GGGGS)3TSEL, the anti-Omalizumab Nb, and a 6xHis tag. To exchange between anti-β-lactam nanobodies, the coding sequence of each nanobody was amplified by PCR using Fw*Sfi*I (5′-3′ seq: GGCCCAGGCGGCCATGG) and Rv*Sfi*I (5′-3′ seq: GGCCGGCCTGGCCTGAGG) and the obtained product was *Sfi*I-digested and cloned into the pINQ-bis vector.

Expression and purification of the Aztreonam/Omalizumab (Az/Om) Calibrator.

E. coli BL21 (DE3) cells were transformed with the pINQ-bis vector and seeded in LB agar−kanamycin plates. LB−kanamycin (200 mL) was inoculated with 2 mL of an overnight culture of a single colony and grown at 250 rpm, 37 °C. When OD600nm = 0.6, IPTG was added to a final concentration of 10 μM and the cells were grown overnight at 28 °C. The following day, periplasmic extracts were prepared as described by Olichon et al.,[20] supplemented with NaCl and imidazole up to 0.3 M and 20 mM, respectively, and the bispecific Nb was purified with Ni-NTA columns using the ÄKTA purification system (GE Healthcare, Uppsala, Sweden) using 50 mM NaH_2PO_4 and 300 mM NaCl pH 7.2 (binding buffer) supplemented with 20 mM imidazole. After washing with binding buffer and 50 mM imidazole, the bispecific Nb was eluted with 250 mM imidazole. The eluted fractions were dialyzed against PBS and concentrated up to 250 μL using a 9K MWCO concentrator (89884A, Thermo Fisher Scientific, Waltham, MA, USA). Two hundred microliters were then purified by size-exclusion chromatography. This second purification step was carried out by injecting 100 μL of the concentrated sample onto a Superose 12 HR 10/30 column (Cat No. 17-0538-01, Amersham, Uppsala, Sweden) run with buffer PBS−0.2% Tween at 0.5 mL/min in the ÄKTA purification system. The MW standard proteins ferritin (440 kDa), aldolase (160 kDa), ovalbumin (45 kDa), α-chymotrypsinogen A (25 kDa), and ribonuclease (14 kDa) were used to build an elution time calibration curve using the UV detector. The bispecific nanobody fraction was kept at −20 °C until use.

Binding analysis of nanobody interactions by biolayer interferometry.

Binding interactions were analyzed using the Blitz system (ForteBio, Inc., Menlo Park, CA). Kinetic rate constants were determined using amine-reactive biosensors (AR2G) according to manufacturer's instructions. Omalizumab was immobilized on the sensors at 30 μg/mL through an N-hydroxysuccinimide/1-ethyl-3-(3dimethylaminopropyl) carbodiimide (NHS/EDC) linkage and then the free amine-reactive sites were blocked with 1 M ethanolamine, pH 8.5. The biosensors were then exposed for 120 s to five different concentrations of Nb E5 and bispecific Nb Az/Om (25, 50, 100, 200, and 400 nM) diluted in PBS with 0.2% Tween 20, 1% BSA, and 0.05% sodium azide (kinetic buffer), followed by a 120 s dissociation step in the kinetic buffer while shaking at 2200 rpm. Interferometry data were globally fitted to a 1:1 binding ratio

for calculating the kinetic parameters using Blitz Pro Software, version 1.2 (ForteBio Inc., USA).

Study of the study of the reactivity of monospecific and bispecific nanobodies.

ELISAs were performed in 96-well microtiter high-binding plates (Cat No. 655061, Greiner Bio-One, Frickenhausen, Germany). All HRP-conjugated reagents (referred in Materials Section) were prepared according to manufacturer's instructions. Samples and reagents were incubated in 100 µL/well PBS−0.1% BSA (dilution buffer) for 1 h at RT with agitation. The peroxidase activity was measured using a substrate solution of TMB and H_2O_2 and absorbance was read at 450 nm with Fluostar Optima reader (BMG, Ortenberg, Germany).

The anti-antigen reactivity of the purified monospecific and bispecific nanobodies was evaluated using ELISA plates coated ith 0.3 µg/well Omalizumab or KLH-conjugated antibiotic. Antigen-bound monospecific nanobodies were detected by incubating wells with anti-HA antibody−HRP or streptavidin−HRP, whereas bispecific nanobodies were detected with anti-His antibody−HRP or by successive steps of 10 µg/mL Omalizumab followed by anti-IgG antibody−HRP. For inhibition ELISAs performed to evaluate the specificity of nanobodies against the paratope of Omalizumab, serial dilutions of nanobodies were coincubated with 1.4 ng/mL Omalizumab in plates coated with 0.1 µg/well IgE. The bound Omalizumab was then detected with anti-IgG antibody−HRP.

The titration curve of the Az/Om calibrator was compared to that obtained with an artificial human serum (cIgE) (Dr.Fooke Laboratorien, Neuss, Germany), which was obtained by combining a fusion of the Fc domain of IgE and FcγRI (CD64) with aztreonam-specific polyclonal rabbit IgG.[7] One unit (1 IU/mL) of the Arthus aztreonam is equivalent to 1 IU/mL of aztreonam-specific IgE.

Analysis of human serum samples containing amoxicillin-specific IgE using the Amoxicillin/Omalizumab (Amx/om) calibrator.

Amx/Om was used as a calibrator in the CLIA luminescence immunoassay for the determination of amoxicillin-specific IgE levels in serum samples. The assay was performed as described by Quintero-Campos et al.[17] Briefly, white flat-bottomed polystyrene ELISA plates were coated with 0.3 µg/well amoxicillin-conjugate, incubated with 25 µL/well serum samples or Amx/Om calibrator for 30 min at RT, and subsequently

incubated with 25 µL/well Omalizumab−HRP solution. The peroxidase activity was measured with chemiluminescent SuperSignal ELISA Femto substrate (Thermo Fisher Scientific, Waltham, MA, USA), and the luminescence signal was read at 450 nm using a multimode plate reader. A calibration curve was built by serial dilutions of the Amx/Om calibrator in IgE-free serum (Cat No. H4522, Sigma, St. Louis, MO, USA) and it was used to quantitate amoxicillin-specific IgE of seven deidentified serum samples from allergic patients. The results were compared to those obtained using the same samples with the ImmunoCAP-specific IgE assay (Thermo Fisher Scientific,Waltham, MA, USA) as a reference method.

RESULTS AND DISCUSSION

Selection of the anti-Omalizumab nanobody

For the generation of the nanobodies (Nbs) to be used for the construction of the recombinant calibrator, a llama was immunized with the Omalizumab antibody and the antigenic determinants of aztreonam and amoxicillin corresponding to the adducts formed through the reaction of their lactam ring with human albumin. After the fourth immunization round, a VH/VHH library of 10^8 transformants was generated from 10^8 blood mononuclear cells. For the selection of the anti-Omalizumab nanobody, in each round of panning, the library was initially incubated in wells of a microtiter plate coated with human IgG1 (the isotype of the Omalizumab antibody) in order to remove the nanobodies that could have been generated against epitopes other than the paratope of Omalizumab. The VHH pool selected from the second round of panning was then cloned en masse into the *Sfi*I sites of the pINQ-BtH6 plasmid for high yield expression and *in vivo* biotinylation. Initially, 89 clones were screened against Omalizumab and selected on the basis of the strength of their reactivity at high dilution ($1/10^7$), which correlates with the relative high affinity/level of expression of the individual clones (Figure S2.1).

Then, 34 of the most reactive clones were tested at a $1/10^2$ dilution against Omalizumab, human IgG1, and total IgG. None of the clones showed cross-reactivity with these antigens indicating that preincubation of the library with human IgG1 efficiently removed clones that were not specific for the Omalizumab paratope (Figure S2.2). Sequencing of 10 of these clones revealed 5 unique sequences (Figure 2.2A), and all of them showed good level of expression (Figure 2.2B,C).

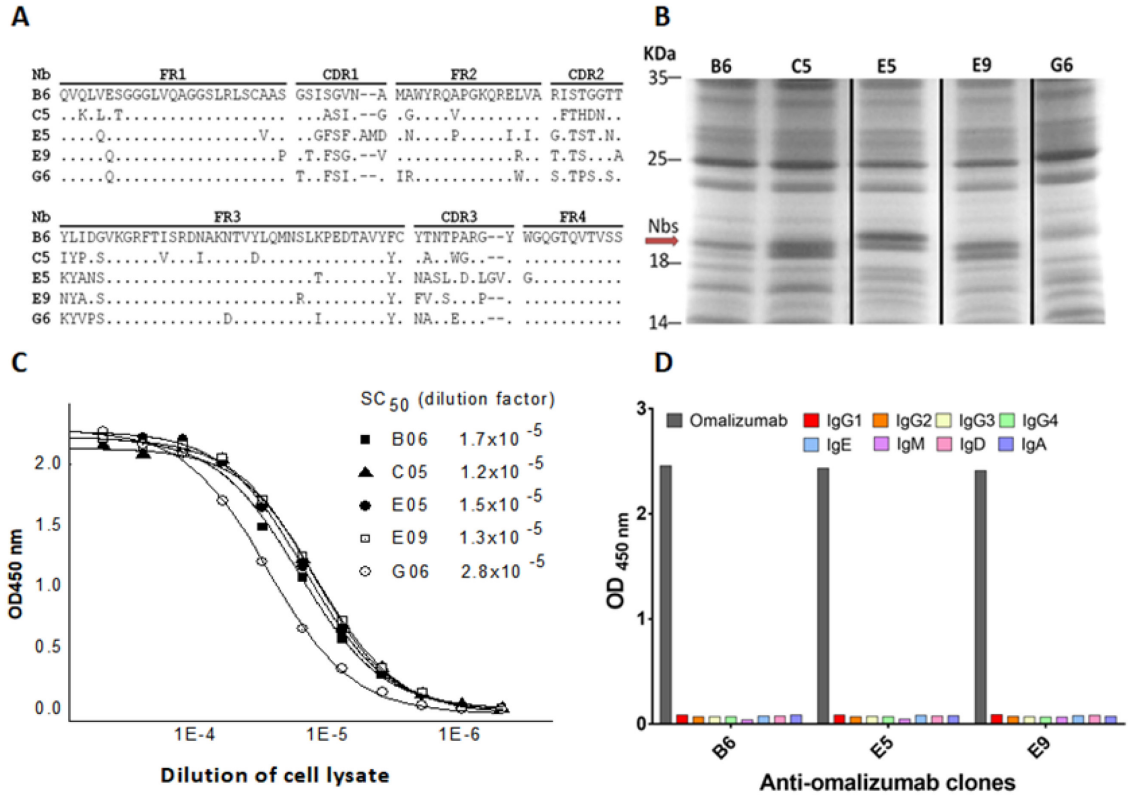

Figure 2.2: Sequences, expression, and reactivity of selected Nb clones. [A] Nb sequence alignment. Dots represent identity to the sequence of B6, and gaps are represented by dashes. Framework (FR) and complementarity-determining regions (CDR) are detailed at the top. [B] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) corresponding to the expression of the Nbs in BL21 cells. [C] Relative expression levels evaluated by reactivity in ELISA against Omalizumab. [D] Reactivity of the Nbs against different classes and subclasses of immunoglobulins was tested at a saturating concentration.

Clones B6, E5, and E9 were expressed, purified by Ni-NTA agarose, and characterized for their reactivity against different classes of immunoglobulins. As shown in Figure 2.2D, the three Nbs showed complete absence of reactivity against all immunoglobulin classes, adding support to the fact that they react against the paratope of Omalizumab (Figure 2.2D), as well as their potential to be used as a diagnostic reagent for clinical samples. The specificity of the three Nbs for the paratope of Omalizumab was confirmed by inhibition tests, which also allowed us to rank the relative affinity of the Nbs, as shown in Figure 2.3. Sub-nanomolar concentrations of the Nbs were sufficient to inhibit 50% of maximum binding (IC50); Nbs B6 and E5 have the highest relative affinity for Omalizumab and the latter was chosen to construct the bispecific calibrators due to its higher level of expression.

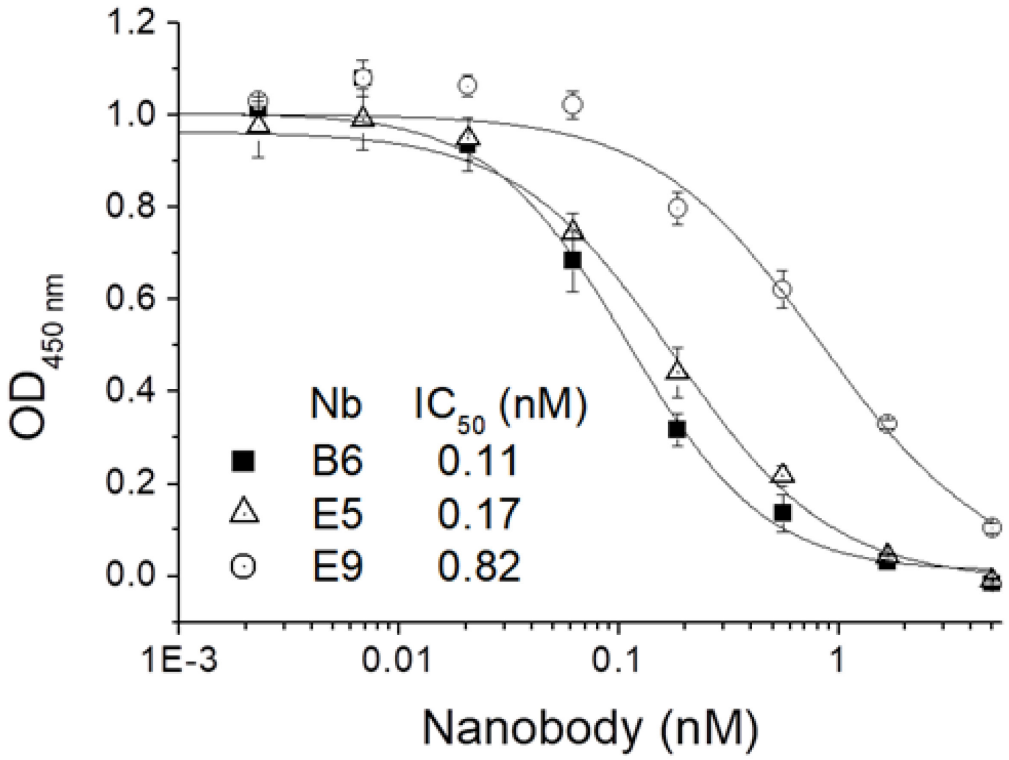

Figure 2.3: Inhibition curves of the interaction of Omalizumab with IgE. The inhibition curves were made by coincubating serial dilutions of the Nbs with fixed amounts of Omalizumab in ELISA plates coated with human IgE and then detecting the binding of Omalizumab with an anti-human IgG−HRP conjugate. IC_{50} values are shown in the inset. Measurements were carried out in triplicate.

Selection of the anti-Aztreonam nanobody

As a first example, a bispecific Nb was generated for the diagnosis of allergies to the antibiotic aztreonam. Selection for anti-aztreonam Nbs was performed from the nanobody library described above on microplates coated with the KLH-conjugated antibiotic. After two rounds of panning, 10 clones were picked. In addition to testing the reactivity of these clones against aztreonam, the cross-reactivity with other β-lactam antibiotics was also evaluated because a nanobody capable of reacting with different antibiotics could allow the same calibrator to function in more than one test (Figure 2.4). In general, there was no cross-reactivity with the carrier protein or with the antigenic determinants of other antibiotics, except in the case of penicillin G, which despite a major structural difference with aztreonam presented some cross-reactivity with many of the clones. However, due to its low intensity, this cross-reactivity with penicillin G would have no practical value, so the anti-aztreonam 01 Nb was finally selected for the construction of the bispecific antibody based on its relative highest affinity/expression level (Figure S2.3).

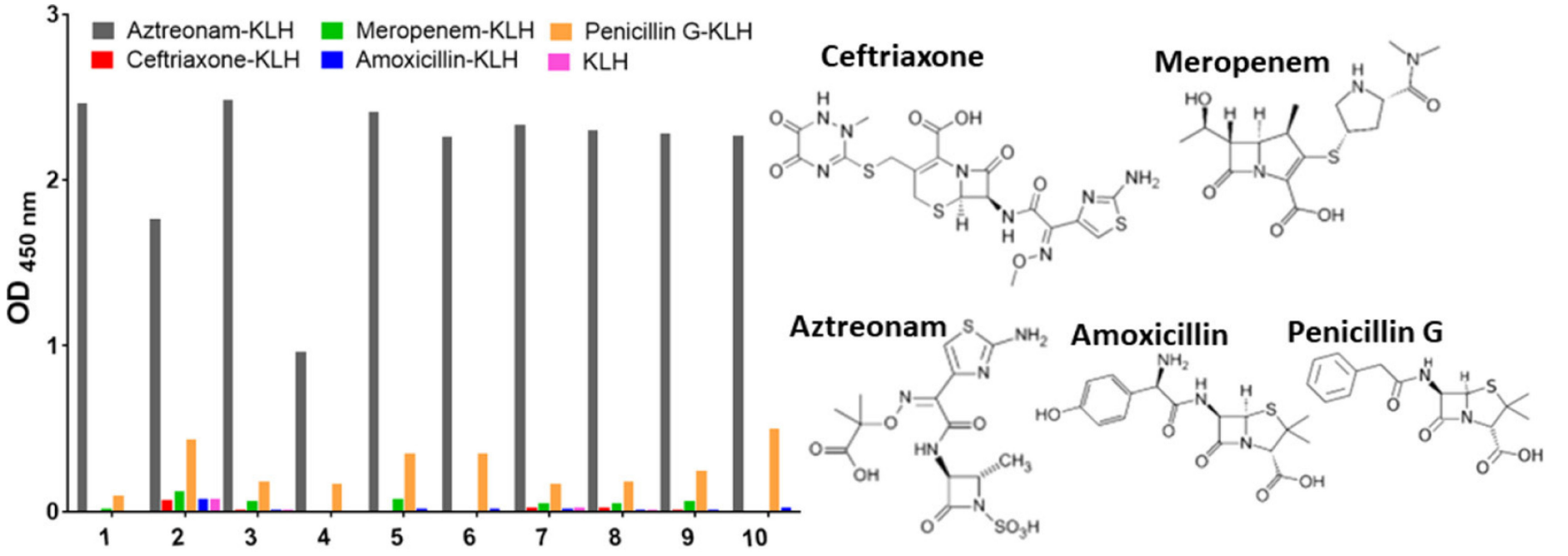

Figure 2.4: Selection of clones expressing anti-aztreonam nanobodies. The supernatants of 10 clones from the second round of panning were tested by ELISA to analyze their reactivity against aztreonam−KLH and cross-reactivity against KLH and other β-lactam antibiotic determinants. The sequence alignment of four of the selected clones is shown below.

Construction of a synthetic calibrator for Aztreonam

The scheme of the expression cassette used for the generation of the bispecific nanobody is shown in Figure 2.5. The bispecific Nb was designed considering the possibility of an adaptable modular use for the diagnosis of allergies to β-lactam antibiotics using the same anti-IgE-detecting antibody (Omalizumab in our case). For this purpose, the nanobody against the Omalizumab paratope was fixed in the C-terminal region of the bispecific antibody, while in the N-terminal region a site was generated for the cloning of the Nb against the target antibiotic (anti-aztreonam in this example). This cloning site is flanked by the two *Sfi*I sites present in the phage nanobody library, so that once the Nb has been selected against the β-lactam antibiotic of interest, it is easily inserted into the expression cassette of the bispecific calibrator.

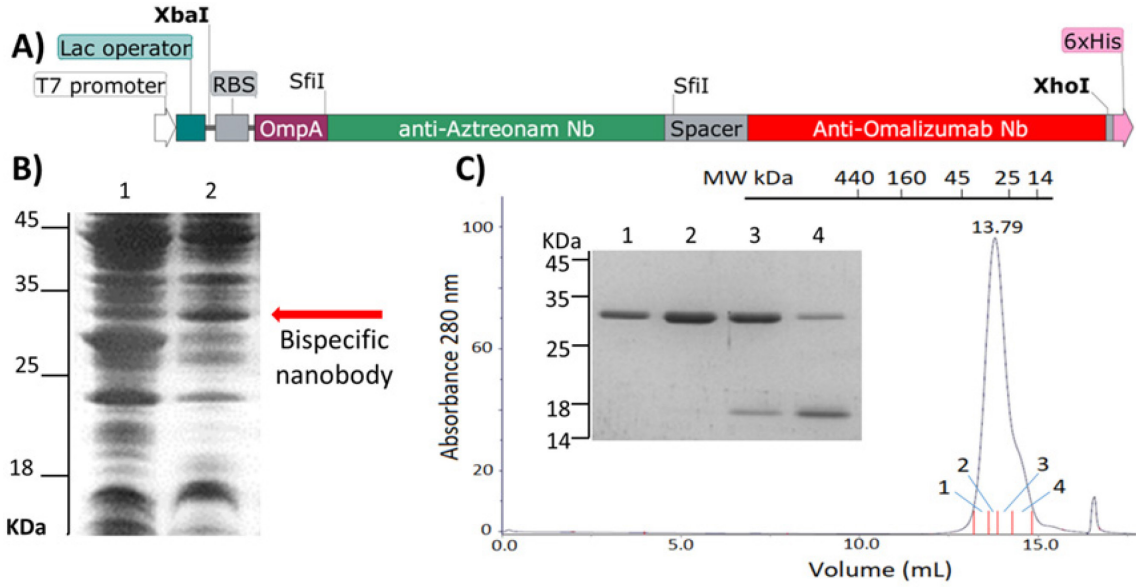

Figure 2.5: Expression and purification of the bispecific nanobody. [A] Cloning cassette inserted between the *Xba*I and *Xho*I sites of pET-28a(+) and used to express the bispecific calibrator (RBS, ribosome binding site; OmpA, signal peptide, and Spacer: GQAGR(GGGGS)3TSEL). [B] SDSPAGE analysis of transformed *E. coli* BL21 soluble cell extract before (1) and after (2) IPTG induction. [C] Gel filtration analysis of the Ni-NTApurified bispecific Nb; the elution volume of the main peak corresponds to the expected molecular size of the calibrator (about 32 kDa). The inset displays the SDS-PAGE analysis of the peak fractions.

The pET-28a(+) plasmid containing the cassette with the 01 Nb to aztreonam and the E5 Nb to Omalizumab was transformed into BL21 *E. coli* cells to produce the aztreonam/Omalizumab (Az/Om) calibrator. The recombinant calibrator was purified from the periplasmic extract and purified by affinity chromatography using Ni-NTA-agarose and gel filtration, Figure 2.5B,C. The Az/Om calibrator was highly pure and did

not associate in solution. There was no, significant loss of reactivity of the individual Nbs in the bispecific construct as observed from the comparison of the titration curves of the monomeric Nbs and the calibrator, Figure S2.4A,B. Considering that the reactivity of the anti-Omalizumab Nb could be affected by the interference of the spacer attached to its N-terminal residue, we further studied the preservation of its reactivity by bio-layer interferometry affinity measurements, Figure S4C,D. There were no substantial changes in the affinity of monomeric (KD = 7.19 nM) or bispecific Nb (KD = 6.97 nM), which is in agreement with the result of the titration curves, and this indicates that the length and flexibility of the spacer do not compromise the recognition of the IgE epitope by the C-terminal Nb.

To simulate the shelf life of the calibrator in the final dilution of use in the assay, an accelerated stability study was carried out. The same calibrator preparation was stored at 4 and 37 °C for 1 and 2 weeks. As shown in Figure 2.6A, there were no significant differences in the performance of the reagent stored under different conditions, confirming that, as expected, the bispecific construct is very stable, which is a very relevant property for its use as a diagnostic reagent. Due to the lack of international standards for aztreonam-specific IgE, the molar equivalence of the Az/Om calibrator in international units of IgE was established based on its correlation with the aztreonam surrogate standard, as shown in Figures S2.5 and 2.6B. On this basis, a 1.87 pM concentration of the Az/Om calibrator corresponds to 1 IU/mL aztreonam-specific IgE. A more direct correlation could be obtained using sera from patients whose specific IgE has been quantified using a reference method. Unfortunately, to the best of our knowledge, there is not yet any *in vitro* commercial assay for the specific determination of IgE to aztreonam.

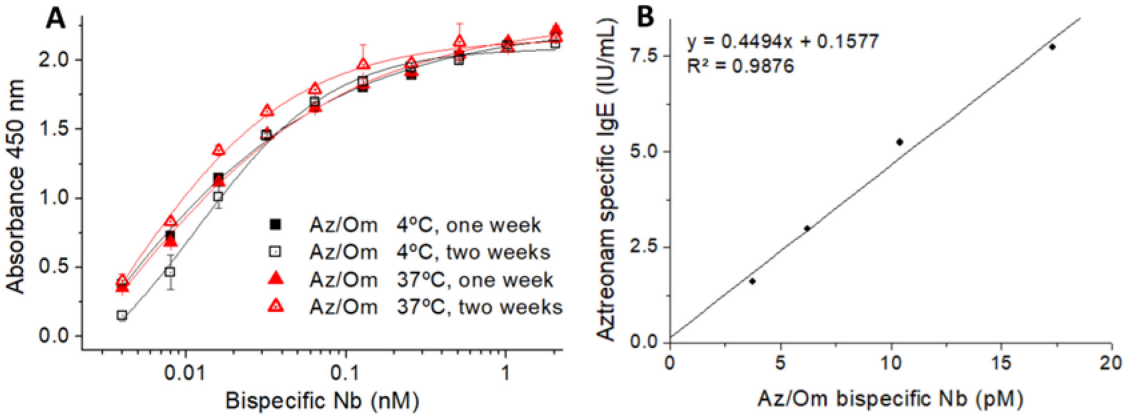

Figure 2.6: Stability and equivalence of the Az/Om calibrator. [A] Accelerated stability of the Az/Om calibrator performed with the bispecific nanobody at the final concentration of use stored at different temperatures. Measurements were carried out in triplicate. Statistical analysis was performed with the Kruskal−Wallis test followed by Dunn's post hoc multiple comparisons test to compare the performance of the reagent stored at different temperatures. [B] Correlation curve of the Az/Om concentration with the surrogate aztreonam-specific IgE standard.

Construction of a synthetic calibrator for Amoxicillin

In the second application, a bispecific antibody was generated for the diagnosis of allergy to amoxicillin. Selection of the anti-amoxicillin Nb was performed in a similar way to that described for aztreonam using the amoxicillin−KLH conjugate. As shown in Figure 2.7A, clones D56, D59, and D10 showed cross-reactivity with all β-lactams tested except for ceftriaxone. These clones could be used to prepare bispecific Nbs that function as a common calibrator for these four antibiotics; however, we preferred to opt for a clone with a higher specificity and chose clone 1, hereinafter called D51, due to its favorable expression. In a similar way to that described for the Az/Om calibrator, this Nb was used to produce the bispecific Nb D51/E5 (Amx/Om).

The bispecific Amx/Om Nb was diluted in IgE-free serum to build a calibration curve on ELISA plates coated with amoxicillin−KLH using HRP-conjugated Omalizumab and the SuperSignal ELISA Femto substrate (Thermo Fisher Scientific), as shown in Figure 2.7B. In parallel, a panel of seven samples of sera from patients with allergy to amoxicillin that had been previously analyzed using the ImmunoCAP system (Thermo Fisher Scientific) was tested using this assay. Figure 2.7C shows that an excellent correlation was obtained between the IU/mL of the amoxicillin-specific IgE concentration measured by the reference technique and the corresponding concentration of the Amx/Om calibrator (Figure 2.7B). Based on this correlation, 259 pM Amx/Om calibrator corresponds to 1 IU/mL amoxicillin-specific IgE.

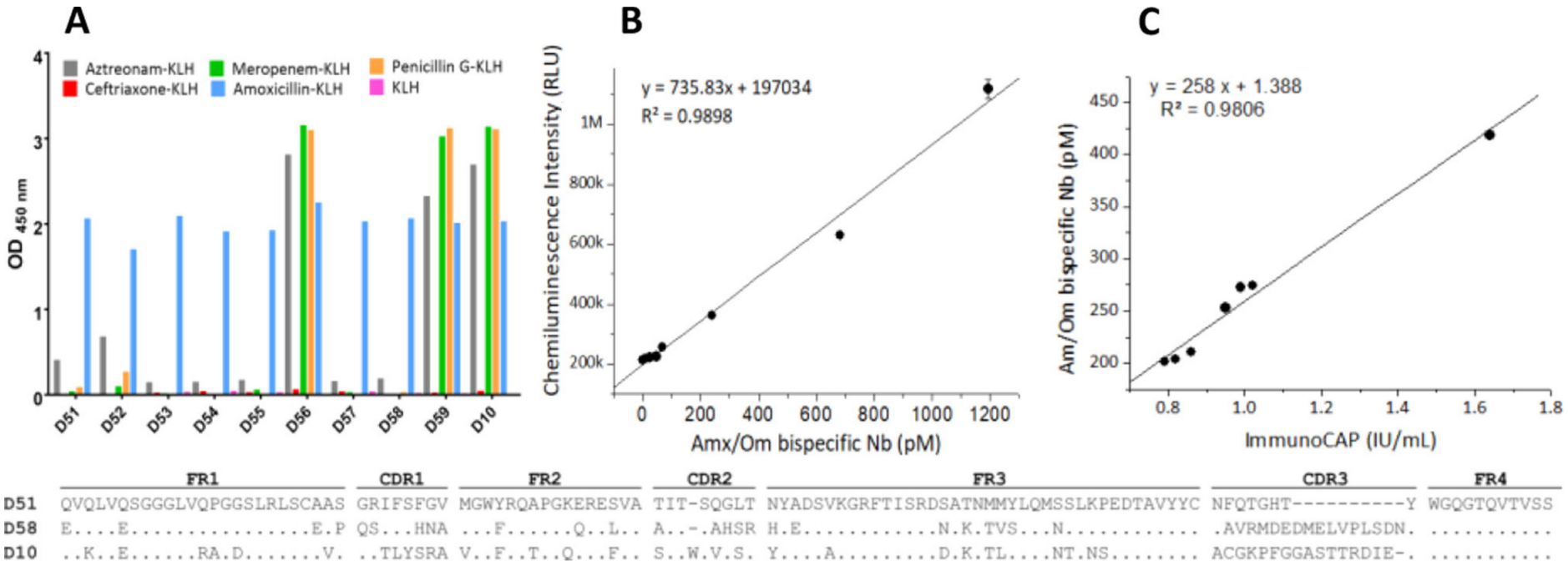

Figure 2.7: Nbs to amoxicillin and performance of the Amx/Om bispecific Nb. [A] Supernatants of 10 clones from the second round of panning were tested by ELISA to analyze their reactivity against amoxicillin−KLH and cross-reactivity against KLH and other β-lactam antibiotic determinants. The sequence alignment of three unique sequences is shown below. [B] Chemiluminescent ELISA, calibration curve obtained with the Amx/Om bispecific Nb. [C] Correlation between the amoxicillin-specific IgE concentration determined by the ImmunoCAP system and the Amx/Om concentration.

CONCLUSIONS

The present study constitutes a proof of concept of the potential use of bispecific nanobody systems as substitutes for calibrators or positive controls based on biological materials for drug allergy testing. At present, the technology for generating nanobodies is highly developed and many laboratories have the capacity to produce them. A key component of the bispecific reagent is a Nb that reacts specifically with the paratope of the secondary antibody used for detection. In practice, due to the comparatively higher immunogenicity of the paratope, obtaining nanobodies with this specificity is undemanding and it can be further facilitated by including a preadsorption step on unrelated antibodies of the same isotype during panning. The other component is a second nanobody that reacts with the target antigen, which is even easier to obtain. Once produced, the bispecific reagent must be purified to homogeneity and its functionality has to be analyzed by ELISA and affinity measurements. Once characterized, the bispecific Nb needs to be calibrated against the reference standard or the reference method, verifying that a linear correlation exists. If this condition is met, the bispecific calibrator has the potential to replace biological standards, without restrictions regarding its nature, be it proteins, lipids, polysaccharides, etc. In addition to their outstanding stability and low-cost production by bacterial fermentation, nanobodies can be indefinitely reproduced in a homogeneous way from their sequence, offering a superb opportunity not only to improve the standardization of allergy tests but also to overcome the difficulties of batch-to-batch reproducibility of many other diagnostic tests.

Finally, it is interesting to note that the use of bispecific Nbs as highly standardized reference materials as shown here can also be extended to other applications. Indeed, for example, in the case of two-site tests for the detection of biomarkers, if the biomarker standard is labile, unstable, difficult to produce or expensive, it could be replaced by a surrogate antigen consisting of a bispecific construct made up of two nanobodies against the individual paratope of the pair of antibodies used in the test. Such a construct could also be useful as a standard in homologous or heterologous agglutination assays.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION:

Bispecific single domain antibodies as highly standardized synthetic calibrators for immunodiagnosis

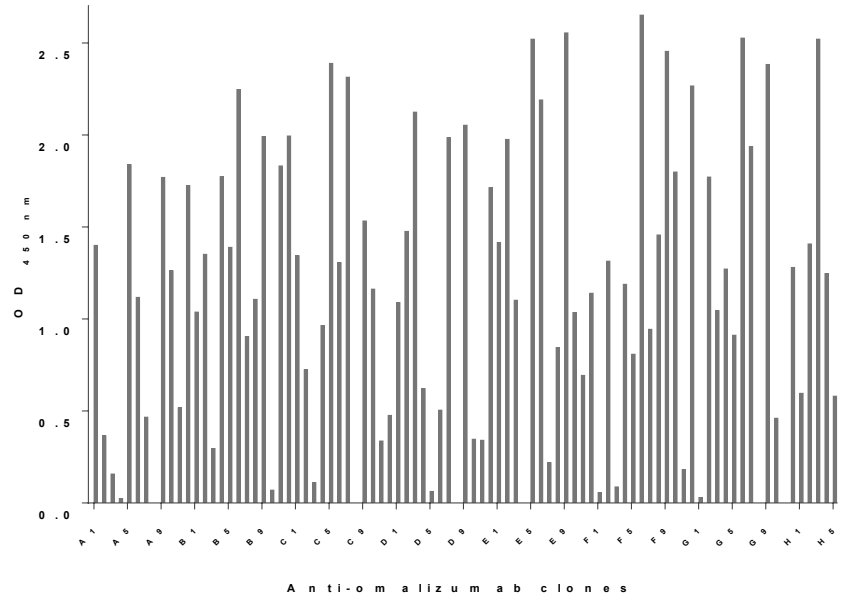


Figure S2.1: Screening of clones expressing anti-Omalizumab nanobodies. Reactivity of soluble cell extracts from bacterial cultures of 84 anti-Omalizumab clones analyzed at a 10^{-7} dilution in ELISA plates coated with the Omalizumab-KLH conjugate.

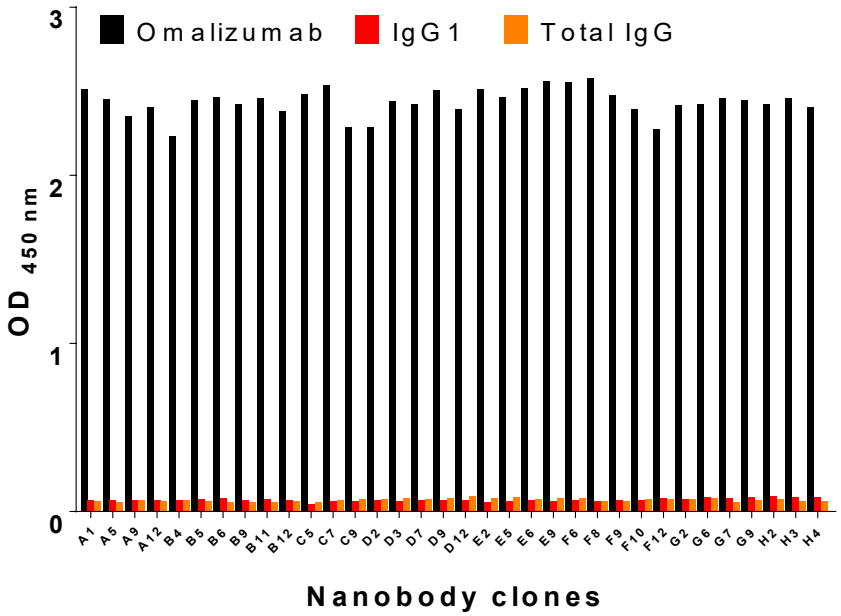


Figure S2.2: Analysis of the specificity of the Nb clones against the paratope of Omalizumab. Thirty four clones were analyzed by ELISA against Omalizumab-KLH, human IgG1, and human total.

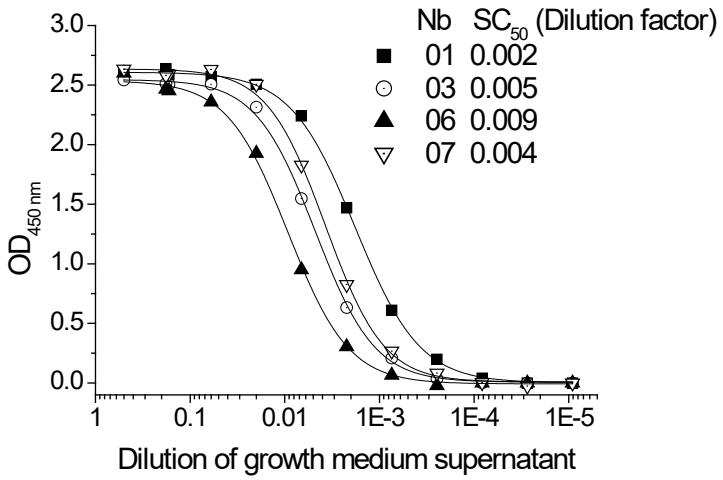
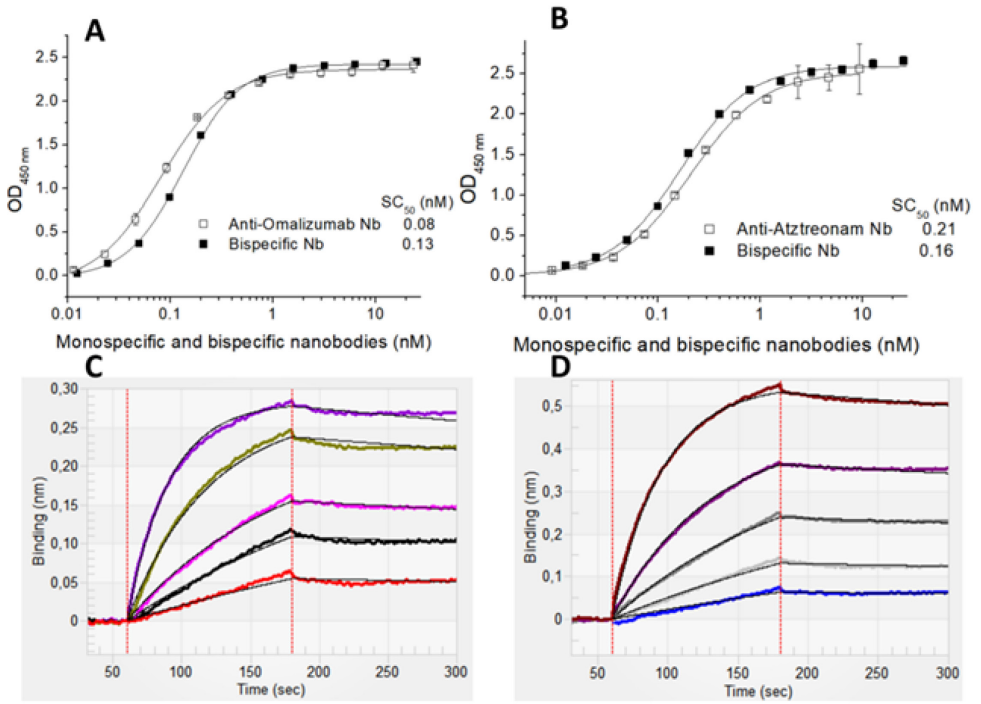


Figure S2.3: Screening of clones expressing anti-Aztreonam nanobodies. Titration curves of bacterial culture supernatants in ELISA plates coated with aztreonam-KLH. The bound nanobody was detected with the anti-His-HRP conjugate.



	K_D (nM)	k_{on} (1/Ms)	k_{off} (1/s)
Nb E5	7.19	8.16×10^4	5.86×10^{-4}
Nb 01/Nb E5	6.97	6.96×10^4	4.85×10^{-4}

Figure S2.4: Preservation of the reactivity of the individual Nb components of the Az/Om calibrator. The antigenic reactivity of the bispecific Nb was compared to that of each of the monospecific Nbs. Titration curves were made by incubating serial dilutions of mono and bispecific nanobodies (triplicates) in ELISA plates coated with Omalizumab [A], or KLH-aztreonam [B] Binding was detected with an anti His-tag antibody coupled to HRP. Binding of the mono [C] and bispecific [D] aztreonam Nb to Omalizumab analyzed by biolayer interferometry (BLI). Bottom) Kinetic parameters of the BLI analysis.

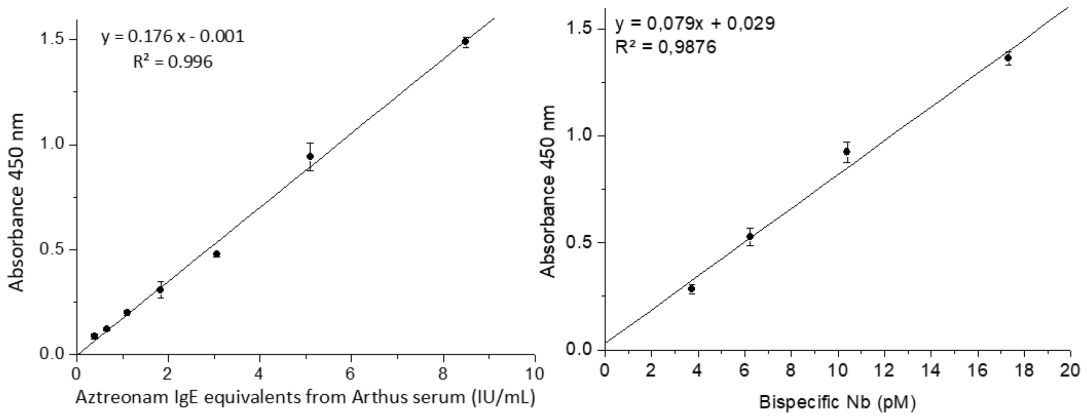


Figure S2.5: The curves were performed by ELISA on plates coated with KLH-aztreonam. Binding of the calibrator was detected by successive incubation steps of 10 µg/mL Omalizumab followed by anti-IgG antibody-HRP.

RESUMEN

La primera parte del capítulo constituye una prueba de concepto del uso potencial de los binanoanticuerpos en pruebas de alergia a medicamentos. La proteína recombinante obtenida está formada por dos nanoanticuerpos, uno encargado de reconocer de manera específica un determinante antigénico de aztreonam o amoxicilina, y otro nanoanticuerpo capaz de unirse selectivamente a un anticuerpo anti-IgE. Se ha caracterizado y se ha comprobado que estas estructuras recombinantes presentan elevada afinidad y selectividad, lo que sumado a su extraordinaria estabilidad y su producción de bajo coste los convierte en unos potenciales calibradores para los ensayos de determinación de IgE específica.

En la segunda parte de este capítulo (**sección 4.2.2**), y teniendo en cuenta las directrices del PROA para el desetiquetado de pacientes alérgicos a penicilina G, se desarrolló un inmunoensayo en el que se utilizaba dicha construcción recombinante como calibrador homólogo en la determinación de sIgE en suero de pacientes alérgicos a este antibiótico, por lo tanto, se abordan los objetivos parciales 2, 4 y 5.

Los resultados obtenidos relacionados con el diseño y producción de proteína recombinante específica a penicilina G son producto del trabajo realizado durante la estancia en el laboratorio del Dr. Gualberto Sapienza. Posteriormente, se estudió la validez del método analizando 70 muestras procedentes de pacientes y controles. Se observó que el método desarrollado lograba una buena concordancia y una fuerte correlación positiva, alcanzando un límite de detección inferior a 0.1 UI/mL y una excelente reproducibilidad (RSD <9%). La sensibilidad clínica del ensayo aumentó significativamente (66%), duplicando la precisión del método de referencia con una especificidad global del 100%. La nueva estrategia de diagnóstico se comparó favorablemente con los resultados obtenidos por el procedimiento estándar, abriendo el camino hacia la estandarización de las pruebas de alergia a la penicilina, y mejorando la sensibilidad de detección de la IgE específica en suero para abordar de forma fiable el desetiquetado de la alergia a los betalactámicos.

4.2.2. An ultra-sensitive homologous chemiluminescence immunoassay to tackle the penicillin allergy

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An ultra-sensitive homologous chemiluminescence immunoassay to tackle penicillin allergy

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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 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.

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 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).[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). The developed immunochemical approach is evaluated by analyzing a cohort of 65 human serum samples.

MATERIALS AND METHODS

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).

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).

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 *Sfi*I, 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 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 h. 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.

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 *Sfi*I 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) and induced with 10 μM IPTG for 16 h 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 was 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.

Biochemical characterization of rBBN.

The purified rBBN was analyzed by 12% sodium ddodecyl sulfate-polyacrylamide 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.

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 min to induce clotting. After centrifugation at 2000 rpm for 15 min, the serum was aliquoted into cryovials and stored at -80 °C 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) 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 (Penicillin Standardised Unit) were determined by interpolating the luminescent signals into the calibration curve.

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 (4 PL) 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.

RESULTS AND DISCUSSION

Phage Display.

The antibody titer of the llama serum (Fig. S3.2) was high after the fourth boost, revealing the production of specific antibodies to PG. Next, a phage-display VHH library with a size of $1 \cdot 10^8$ 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 Fig. S3.3, 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.

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. Fig. 3.1A 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, Fig. 1B) was observed, corresponding to the synthetic standard with an expected molecular weight of around 35 kDa.

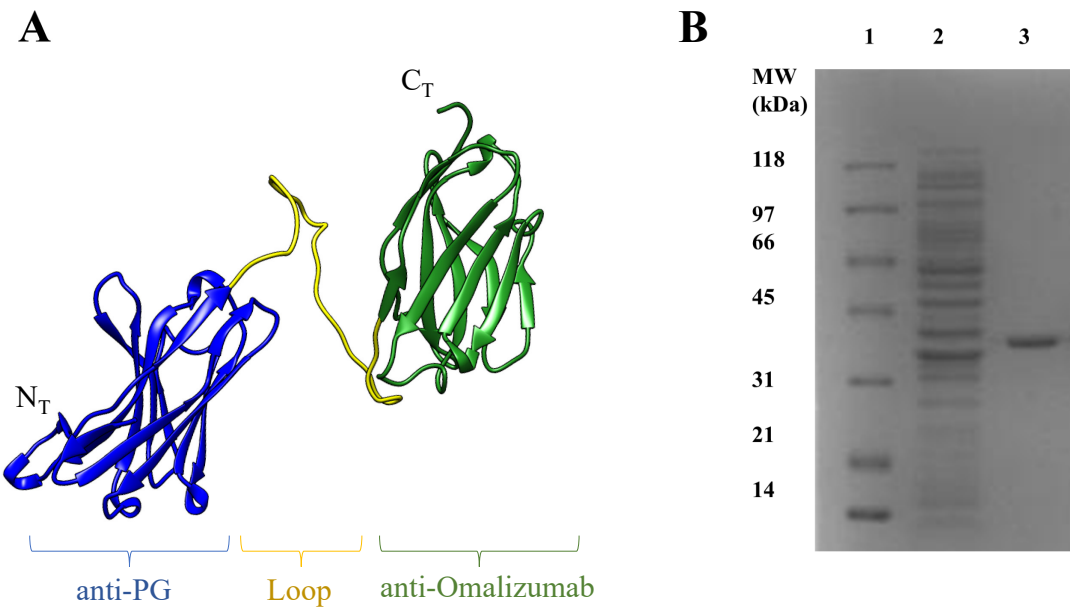

Figure 3.1: [A] Model structure of the rBBN. On the left (blue), the nanobody that targets the antigenic determinant, on the right (green) the one that binds the paratope of Omalizumab. [B] Purification results 12 % SDS-PAGE: (1) Ladder (2) Purification Flow Through (3) Bispecific binanobody with a molecular weight of 35 kDa.

Biochemical characterization of rBBN.

The functionality of rBBN was tested by ELISA, following the protocol described in the supporting information. As shown in Fig. 3.2A, 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 (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 Fig. 3.2B, 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.

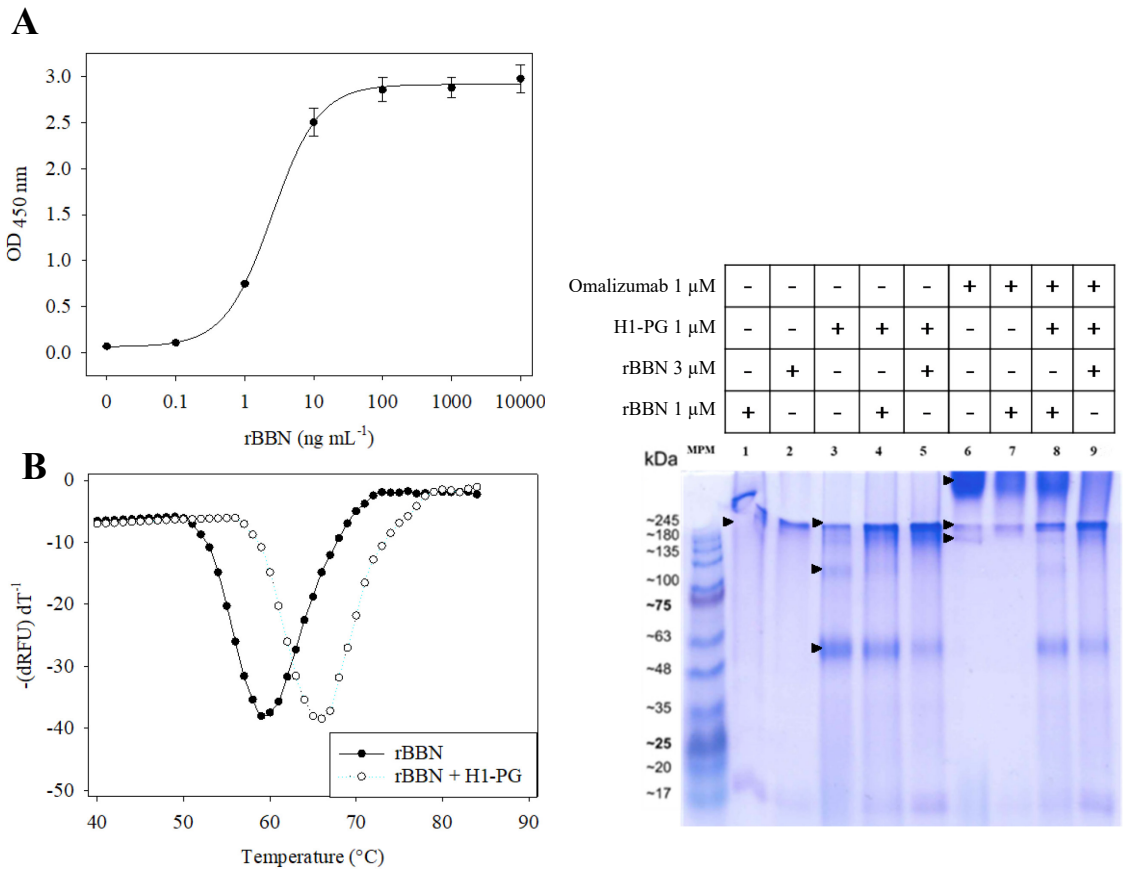

Figure 3.2: [A] Curve of functionality of the bispecific binanobody. [B] Results of thermofluor assay for rBBN alone and bound to conjugate H1-PG. Representation of the melting curve using the first derivative –(dRFU)/dT of the raw data. The Tm is the apex. [C] Blue Native Page: (1) rBBN 1 µM. (2) rBBN 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.

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. Fig. 3.2C 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 min. 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.

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 (Fig. 3.3A) in the hmCLIA method. The current method for the determination of sIgE follows a heterologous calibration and defines 1.0 IU/mL is equivalent to 2.4 ng/mL 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 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. 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.

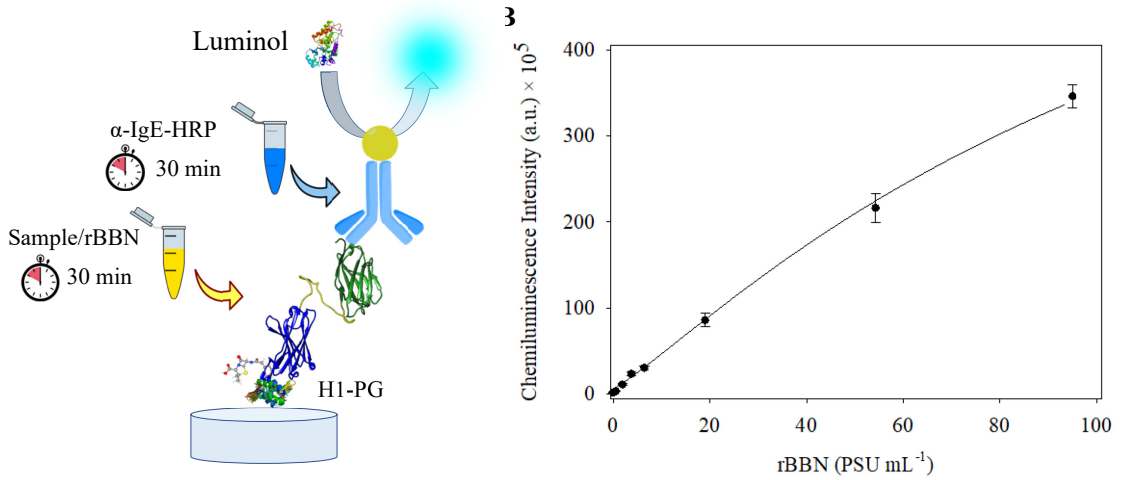

Figure 3.3: [A] Scheme of the homologous chemiluminescence immunoassay for the determination of specific IgE to penicillin G in human serum, using the rBBN as calibrator. [B] Binanobody calibration curve.

Fig. 3.3B 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 and a LOD of 0.02 PSU/mL (8.8 pg /mL), a concentration below the current internationally accepted cut-off for allergy diagnosis (0.1 IU/mL or 240 pg/mL). In the hmCLIA accuracy assessment, the CVs raranged from 2–9%, 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 (Fig. 3.4A) 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 ($r = 0.999$, $p < 0.0001$) (Fig. 3.4B). In addition, as shown in Fig. 3.4B, the slope of the curve is 3, representing the relationship between the value given by ImmunoCAP and that obtained using rBBN as standard.

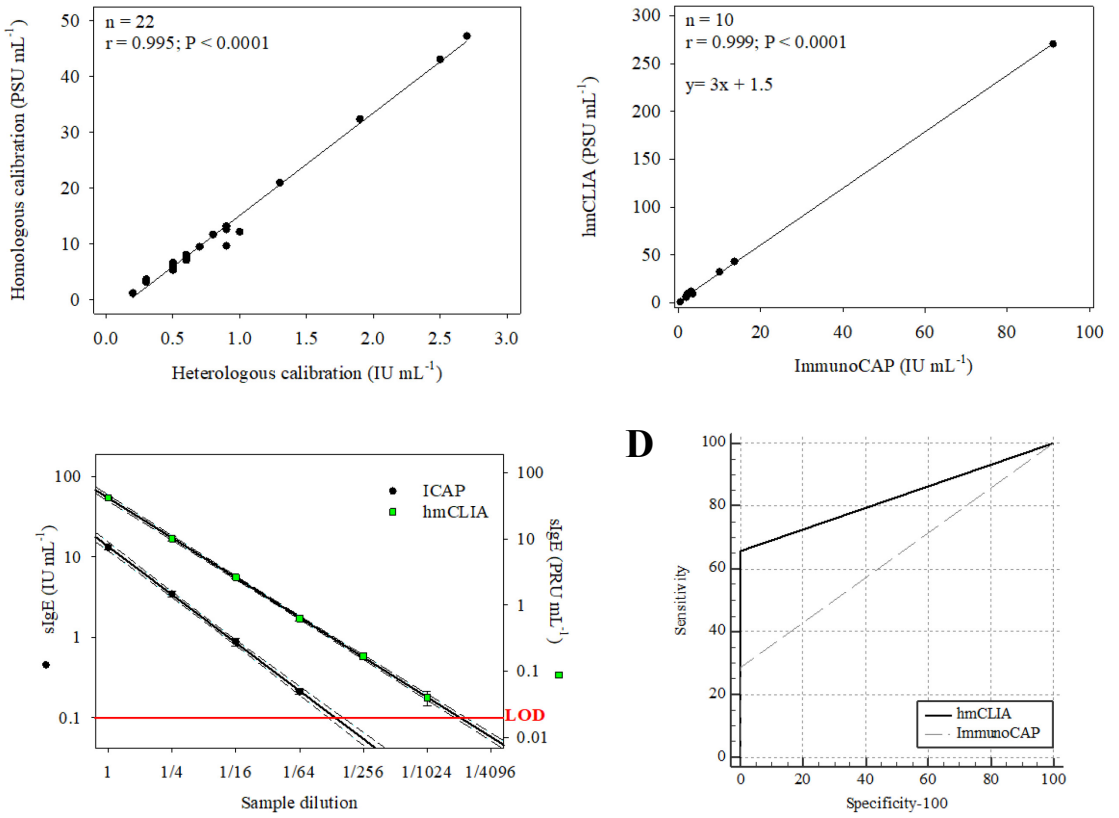

Figure 3.4: [A] Scatter diagram and regression line of inter-method comparison between homologous calibration (rBBN) and heterologous calibration (WHO). [B] Scatter diagram and regression line of inter-method comparison between hmCLIA and ImmunoCAP. [C] Dilution linearity study, using the pooled serum 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).

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 3.1). 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 Fig. 3.4C, 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) 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 (nonallergic 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. It is worth mentioning that all the positive results given by ImmunoCAP were also positive by hmCLIA. Receiver Operating Characteristic (ROC) (Fig. 3.4D) 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.

Table 3.1: Specific IgE to Penicillin G determined by hmCLIA and ImmunoCAP (ICAP).

Patient	CALIBRATION				Recovery (%)
	WHO		rBBN		
	ICAP (IU/mL)	CLIA (IU/mL)	hmCLIA (PSU/mL)	hmCLIA[a] (PSU/mL)	
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	< 0.06	< 0.02	--
4	13.6	2.5 ± 2.5	43.1 ± 0.7	14.4 ± 0.2	106
5	< 0.1	< 0.1	< 0.06	< 0.02	--
6	< 0.1	< 0.1	< 0.06	< 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	< 0.06	< 0.02	--
11	< 0.1	< 0.1	< 0.06	< 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	< 0.06	< 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	--

Patient	CALIBRATION				Recovery (%)
	WHO		rBBN		
	ICAP (IU/mL)	CLIA (IU/mL)	hmCLIA (PSU/mL)	hmCLIA[a] (PSU/mL)	
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	< 0.06	< 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	< 0.06	< 0.02	--
26	< 0.1	< 0.1	< 0.06	< 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	< 0.06	< 0.02	--
30	< 0.1	1.3 ± 0.1	21.0 ± 0.2	7.0 ± 0.1	--
31	< 0.1	< 0.1	< 0.06	< 0.02	--
32	< 0.1	< 0.1	< 0.06	< 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[b]	12.5	4.5 ± 0.4	41.1 ± 3.8	13.3 ± 1.1	106

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

CONCLUSSIONS

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 interlaboratory 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.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Pedro Quintero-Campos: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Paula Segovia-de los Santos:** Investigation. **Ethel Ibáñez-Echevarria:** Conceptualization, Investigation, Resources, Data curation. **Dolores Hernández-Fernández de Rojas:** Resources, Data curation. **Patricia Casino:** Investigation, Methodology, Data curation, Formal analysis. **Gabriel Lassabe:** Conceptualization, Methodology, Data curation. **Gualberto González-Sapienza:** Conceptualization, Methodology, Data curation. **Ángel Maquieira:** Conceptualization, Project administration, Funding acquisition. **Sergi Morais:** Conceptualization, Methodology, Supervision, Project administration, Writing – original draft.

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SUPPORTING INFORMATION

An ultra-sensitive homologous chemiluminescence immunoassay to tackle penicillin allergy

1. Characterization of antigens

HSA and histone (H1) antigens were characterized by SDS-PAGE electrophoresis. As shown in Figure S3.1, there were no significant differences in the molecular weight between unconjugated protein and the antigens produced at molar ratios of 100 and 300. However, slight molecular weight variations were observed for the antigens produced at a molar ratio of 1000, corresponding to approximately 40 kDa. This molecular weight variation corresponded to ten penicillin molecules conjugated per molecule of HSA.

Regarding H1 antigens, different bands were displayed for the raw protein around 30 kDa because H1 is a pool of six isomers with similar primary sequences. A low conjugation was observed at molar ratios 30 and 100, but a high molecular weight variation was observed for the antigens produced under higher molar ratios. Indeed, the molar ratio of 300 varied the molecular weight is approximately 10 KDa, resulting in 25–30 molecules of penicillin G per molecule of H1. The molecular weight variation was more evident for the antigen prepared with a molar ratio of 1000.

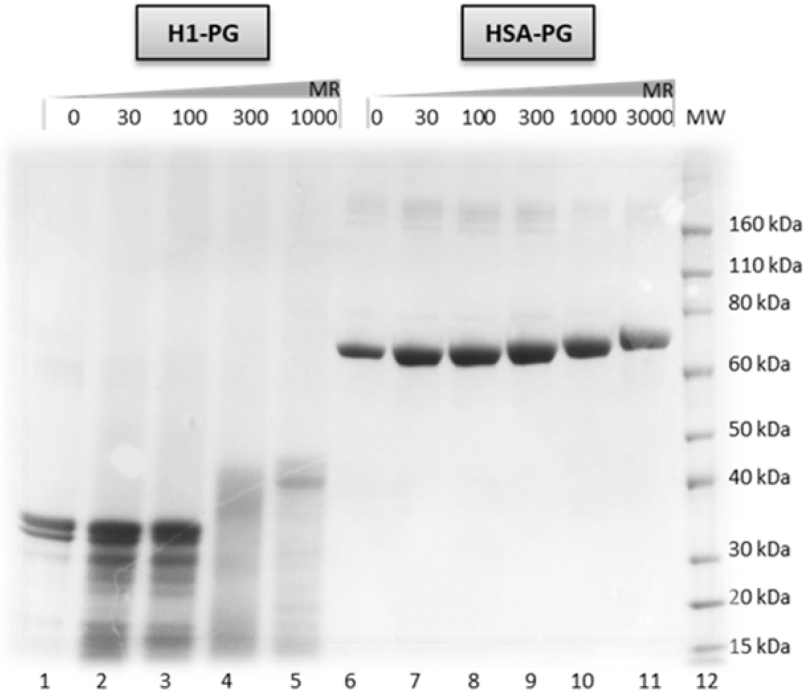


Figure S3.1: SDS-PAGE analysis of H1-PG and HSA-PG antigens. Lines 1, 2, 3, 4, and 5 correspond to H1-derived antigens, and 6, 7, 8, 9, 10, and 11 to HSA antigens. Line 12 is the ladder market (15-160 KDa).

2. Results of the immunization

The reactivity of the serum throughout the immunization process (Figure S3.2) shows that after the third booster dose (extraction 4), the antibody titer was high, confirming the generation of the antibodies of interest by the immune system of the llama.

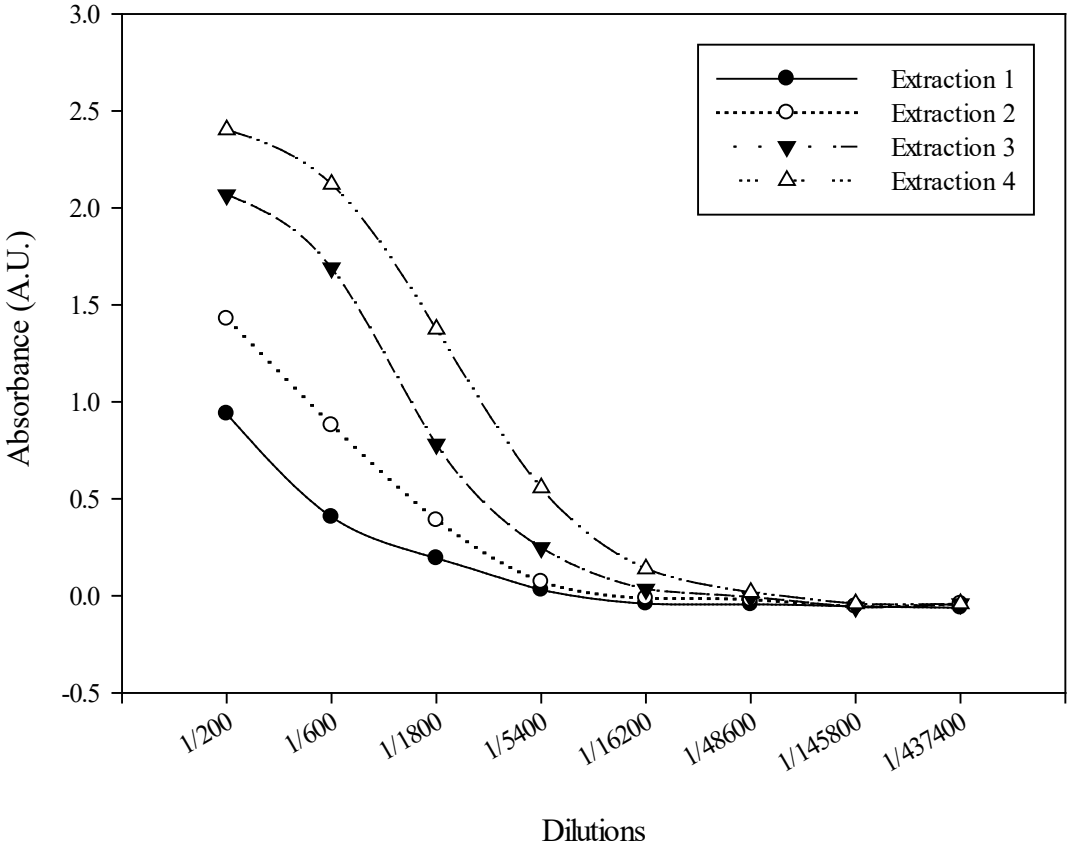


Figure S3.2: Results of the antibody titer to penicillin G.

3. Phage display methodology

3.1.Library construction

According to the manufacturer's protocol, the lymphocytes from the immunized llama were purified by gradient centrifugation with Histopaque (Sigma). From 10^8 cells, total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA USA) and retrotranscribed to cDNA using the RevertAID Reverse Transcriptase (ThermoFisher Scientific, Carlsbad, CA, USA). Subsequently, by PCR and using the forward primers VH1, VH3, VH4, and the reverse primer JH, the DNA fragments encoding the VH/VHH of the IgGs were amplified. The fragments obtained were digested using *Sfi*I and purified by agarose gel electrophoresis. Then, the fragments were cloned into the pComb3X phagemid vector (from Dr. Barbas, The Scripps Research Institute, La Jolla, USA). The ligated product was electroporated into *E. coli* ER2738 competent cells. Finally, cells were inoculated in 10 mL of LB containing 100 μg/mL ampicillin and incubated for 2 h, at 37°C and shaking, before being superinfected with the helper phage M13KO7. After 30 min without shaking, kanamycin (50 μg/mL) was added, and the cells were cultured overnight. The supernatant was harvested by centrifugation. The phages precipitated with 0.2 volumes of 20% polyethylene glycol 8000 in 2.5 M NaCl and resuspended in phosphate-buffered saline (PBS) BSA (3%) and complete EDTA-free protease inhibitor cocktail. The VH/VHH phage library was filtered through 0.22 μm and stored at - 80 °C.

3.2.Panning strategy for the selection of nanobodies

High-binding 8-well strips (Greiner Bio-One, Monroe, NC) were coated with 100 μL/well of 5 μg/mL KLH-penicillin G conjugate in phosphate-buffered saline (PBS) at 4 °C for 16 hours. The wells were blocked with PBS 1% BSA for 30 minutes at 37 °C, followed by washing with PBS-T 0.05%. The coated wells were then incubated with $2 \cdot 10^{10}$ phage particles/well of the VH/VHH library for 1 hour at room temperature (RT) and gentle agitation. The wells were washed 10 times with PBS-T and incubated with PBS-T for 30 minutes at RT with shaking. The wells were washed as before, and the bound phages were eluted by incubation with 50 μL/well of trypsin at 10 mg/mL for 30 min at 37 °C. Finally, the phages were collected and used for titration and subsequent amplification in *E. coli* ER2738 for an additional round of panning. Two rounds were performed.

3.3.Screening of positive clones by ELISA

Once the panning rounds were completed, 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 ampicillin at 37°C and shaken 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 the cultures were incubated with shaking at 37 °C. After 16 hours, the cultures were centrifuged, and the supernatant containing the nanobodies was isolated.

An ELISA plate was coated with 5.0 µg/mL of KLH-Penicillin G conjugate in PBS for 16 hours at 4 °C. After blocking with 1% PBS-BSA for 30 minutes at 37 °C, the plate was washed 4 times with PBS-T. Next, 100 µL/well of a 1:2 dilution of each of the supernatants of the selected clones were dispensed in 0.1% PBS-BSA and incubated for 1 hour at RT. Subsequently, after washing the plate, a solution of peroxidase-conjugated anti-HA antibody was incubated under the same conditions. Finally, after washing, positive clones were detected by the enzymatic activity developed for 15 min at RT by adding 100 µL/well of HRP substrate (0.4 mL of 6 mg/mL solution of 3,3′,5,5′-tetramethylbenzidine in DMSO, 0.1 mL of 1 % H_2O_2 in water, in a total of 25 mL of 0.1 M acetate buffer, pH 5.5). The enzymatic reaction was stopped by adding 50 µL of 2N H_2SO_4, reading the absorbance at 450 nm on a Fluostar Optima plate reader (BMG, Ortenberg, Germany).

4. Protocols for the biochemical characterization of rBBN

4.1.ELISA assay protocol to evaluate the functionality of the rBBN

An ELISA plate was coated for 16 hours at 4 °C with 5.0 µg/mL of the KLH-Penicillin G conjugate in PBS. After blocking with PBS-BSA 1% for 30 minutes at 37 °C, the plates were washed 4 times with PBS-T. Next, 100 µL/well of 1:10 serial dilutions (starting from 10 µg/mL) of rBBN were dispensed in PBS-BSA 0.1% and incubated for 1 hour at RT with shaking. Subsequently, after washing the plate, 100 µL/well of 10 µg/mL Omalizumab was incubated under the same conditions. Next, after washing the plate as before, 100 µL/well of anti-human-IgG-HRP (1:20,000) was dispensed and incubated under the same conditions. Finally, the result was detected after washing the wells by adding the HRP substrate solution.

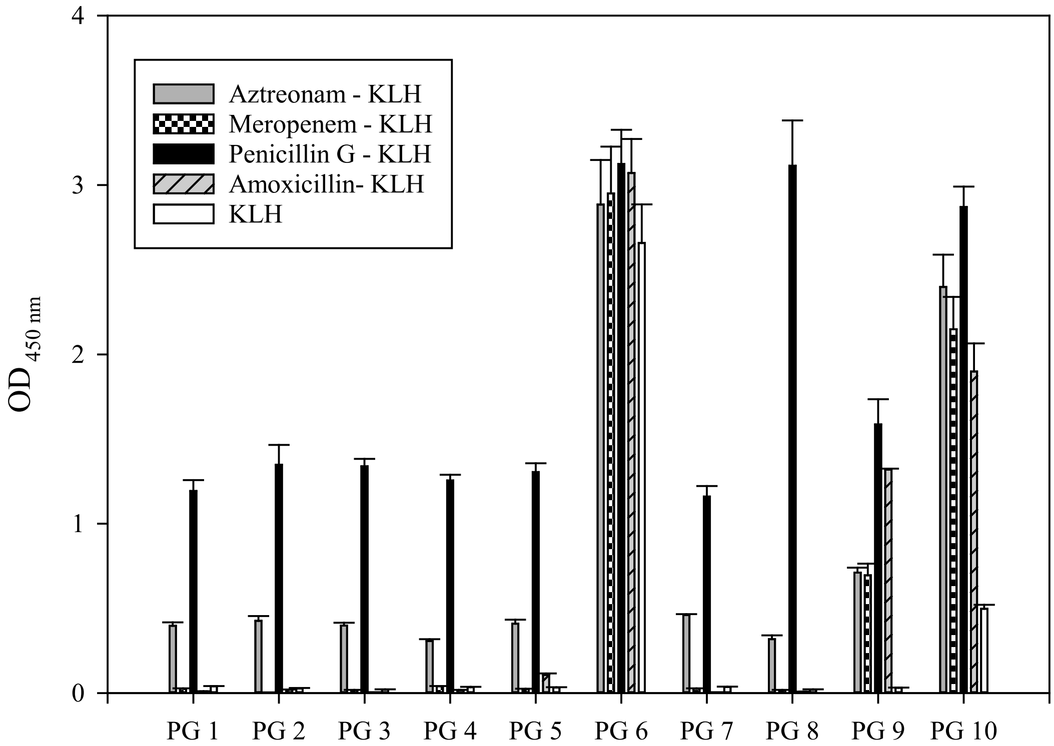
4.2.Thermofluor

Thermofluor experiments were performed using a CFX Connect Real-Time PCR System unit (Bio-Rad). The samples were prepared with 10 µM rBBN and 10 µM PG-conjugate in 50 mM HEPES pH 7, 150 mM NaCl buffer. The protein alone was studied, and the protein with penicillin G conjugate. The (5000x) SYPRO Orange concentrated dye was added to each sample at a final ratio of 15x concentration.

Samples were incrementally heated from 20 to 90 °C at a rate of 1 °C/min. The fluorescence intensity at each temperature was recorded at 510–530 nm, following excitation at 450–490 nm. The emission intensity was recorded as a function of temperature and was used to obtain a negative first derivative plot. The temperature for the maxima was determined, which corresponds to the protein's melting temperature. All experiments were repeated in triplicate.

5. Selection of nanobodies

The selectivity of the nanobodies was studied, using a panel of conjugates to estimate the affinity of the clones against different conjugates and the carrier protein (Figure S3.3). The specific clones showed slight cross-reactivity with the aztreonam conjugate; however, the signal obtained was almost negligible compared to the signal obtained for the penicillin G conjugate. After sequencing the clones, PG8 was selected for further studies.



Nb	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
PG8	QVQLQESGGGLVQPGGSLRLSCAAS	GFMFSSYA	MSWVRQAPGKGLEWVS	GISSGGNMR	S-ADSVKGRFAISRDNAENTLYLQMFNLKPEDTAVYFC	AKYQTAWVRSGNEYEYEY	RGQGTQVTVSS
PG2	....VQ....................	..T....T	....	A.N...GAT	.Y........T......K.......NS.........Y.	..VIG--IKE.S-----.	W..........
PG3	..K.V....D................	..T..I.G	.TR...........L.	RMDNS.GKT	TYTG......T......K.....L.NS.R...A.D.Y.	..LP.TCSLANS---MD.	W.K..H.....

Figure S3.3: Results of screening for Penicillin G conjugate. The conjugates used in this assay were Aztreonam (AZT), Meropenem (MRP), Penicillin G (PG), and Amoxicillin (AMX) linked to the KLH carrier protein. All conditions were analyzed in duplicate. The sequence alignment of three of the selected clones is shown below.

5. Clinical characteristics of subjects in the case study

Table S3.1: Clinical history of allergic patients

Patient	Culprit Drug	Sex	Age	Allergen
1	Augmentine	M	74	Ring
2	Penicillin	M	61	Ring
3	Amoxicillin	F	53	Ring
4	Augmentine	M	52	Ring
5	Augmentine	M	71	Ring
6	Augmentine	F	59	Ring
7	Augmentine	F	61	Ring
8	Cefuroxime	F	59	Ring
9	Augmentine	M	85	Side Chain
10	Augmentine	M	49	Ring
11	Augmentine	M	49	Ring
12	Augmentine	F	38	Ring
13	Penicillin	M	61	Ring
14	Augmentine	M	70	Side Chain
15	Amoxicillin	F	58	Ring
16	Augmentine	M	72	Side Chain
17	Cefuroxime	F	59	Ring
18	Augmentine	M	70	Side Chain
19	Augmentine	M	74	Ring
20	Augmentine	F	73	Ring
21	Amoxicillin	F	54	Side Chain
22	Augmentine	F	36	Ring
23	Augmentine	F	38	Ring
24	Augmentine	F	61	Ring
25	Augmentine	M	49	Ring
26	Augmentine	M	42	Ring
27	Amoxicillin	M	39	Ring
28	Augmentine	F	39	Ring
29	Augmentine	F	49	Ring
30	Augmentine	F	38	Ring
31	Cefuroxime	F	36	Ring
32	Cefuroxime	F	75	Ring
33	Penicillin	M	61	Ring
34	Augmentine	F	61	Ring
35	Augmentine	M	70	Side Chain

M: Male, F: Female.

6. Results of the controls study

Table S3.2: Specific IgE to Penicillin G determined in serum control samples by hmCLIAand ImmunoCAP (ICAP).

			Calibration			
			WHO	WHO	Binanobody	
Patient	**Sex**	**Age**	**ICAP (IU mL^{-1})**	**CLIA (IU mL^{-1})**	**hmCLIA (PSU mL^{-1})**	**hmCLIA* (PSU mL^{-1})**
36	M	21	< 0.10	< 0.10	< 0.06	< 0.02
37	F	32	< 0.10	< 0.10	< 0.06	< 0.02
38	M	55	< 0.10	< 0.10	< 0.06	< 0.02
39	F	35	< 0.10	< 0.10	< 0.06	< 0.02
40	F	79	< 0.10	< 0.10	< 0.06	< 0.02
41	F	71	< 0.10	< 0.10	< 0.06	< 0.02
42	F	64	< 0.10	< 0.10	< 0.06	< 0.02
43	F	60	< 0.10	< 0.10	< 0.06	< 0.02
44	M	82	< 0.10	< 0.10	< 0.06	< 0.02
45	F	56	< 0.10	< 0.10	< 0.06	< 0.02
46	M	62	< 0.10	< 0.10	< 0.06	< 0.02
47	M	63	< 0.10	< 0.10	< 0.06	< 0.02
48	M	76	< 0.10	< 0.10	< 0.06	< 0.02
49	M	56	< 0.10	< 0.10	< 0.06	< 0.02
50	M	46	< 0.10	< 0.10	< 0.06	< 0.02
51	F	69	< 0.10	< 0.10	< 0.06	< 0.02
52	F	35	< 0.10	< 0.10	< 0.06	< 0.02
53	F	42	< 0.10	< 0.10	< 0.06	< 0.02
54	M	31	< 0.10	< 0.10	< 0.06	< 0.02
55	F	61	< 0.10	< 0.10	< 0.06	< 0.02
56	F	71	< 0.10	< 0.10	< 0.06	< 0.02
57	M	63	< 0.10	< 0.10	< 0.06	< 0.02
58	F	66	< 0.10	< 0.10	< 0.06	< 0.02
59	F	49	< 0.10	< 0.10	< 0.06	< 0.02
60	F	50	< 0.10	< 0.10	< 0.06	< 0.02
61	F	22	< 0.10	< 0.10	< 0.06	< 0.02
62	M	68	< 0.10	< 0.10	< 0.06	< 0.02
63	F	58	< 0.10	< 0.10	< 0.06	< 0.02
64	F	45	< 0.10	< 0.10	< 0.06	< 0.02
65	F	72	< 0.10	< 0.10	< 0.06	< 0.02

M: Male; F: Female.

*The concentrations are multiplied by a factor of 1/3 to get those obtained by ImmunoCAP.

4.3. CAPÍTULO 3

Producción de IgE sintética con reactividad a β-lactámicos

RESUMEN

En el **capítulo 2** se describe la producción de una proteína recombinante formada por dos nanoanticuerpos que estructuralmente se comporta como una sIgE a antibióticos BLCs. El binanoanticuerpo específico a penicilina G se utiliza en un inmunoensayo para realizar un calibrado homólogo y determinar, siguiendo este esquema, la concentración de sIgE a penicilina G en muestras procedentes de paciente alérgicos a este antibiótico. Se consigue alcanzar buena sensibilidad analítica (0.02 PSU/mL) conservando la especificidad clínica (100%) y aumentando la sensibilidad clínica al 66%. De manera que se consigue realizar una calibración homóloga donde se tienen en cuenta todos los componentes del inmunoensayo.

En el presente **capítulo 3** se abordan los objetivos parciales 3, 4 y 5, y se avanza un paso más describiendo la producción de una IgE específica humana recombinante (rsIgE). Para su obtención se recurrió a la combinación de dos metodologías: Phage Display y al sistema de expresión de baculovirus en células de insecto. Para conseguir una inmunoglobulina de origen genómico completamente humano, se utilizó como material de partida la sangre de un paciente alérgico a amoxicilina y penicilina G. A partir del material genético aislado, se obtuvo una biblioteca inmune con la que se realizó Phage Display y se aislaron los clones que expresaban scFv específicos a amoxicilina y penicilina G. Posteriormente, mediante ingeniería de anticuerpos y métodos de expresión en células de insecto se consigue producir dicha rsIgE.

De esta manera, se obtiene un producto recombinante que cumple con los requisitos para su adopción como material de referencia en ensayos *in vitro*: estabilidad, especificidad, seguridad biológica y capacidad de producción en masa. Esta proteína recombinante satisface la necesidad que existe actualmente de disponer de patrones o material de referencia específicos con el que validar nuevos ensayos, así como para poder realizar una estandarización de los métodos existentes.

Igualmente, esta rsIgE se ha utilizado como calibrador sintético para llevar a cabo una calibración homóloga, evaluando las prestaciones del ensayo mediante la cuantificación de sIgE de una cohorte de 150 muestras representativas, y comparando los resultados con el método de referencia.

4.3.1. Production of a human synthetic IgE to develop highly sensitive *in vitro* diagnostic testing for β-lactam antibiotic allergy

Production of a human synthetic IgE to develop highly sensitive *in vitro* diagnostic testing for β-lactam antibiotic allergy

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ESTADO: Enviado a Clinical Chemistry

ABSTRACT

Background: There are no well-validated assays for β-lactam allergy *in vitro* testing. The diagnostic methods must be validated internationally by laboratory proficiency tests with human serum samples. The lack of availability of β-lactam IgE antibody pools of patient sera with defined selectivity prevents standardized methods. Consequently, synthetic IgEs reacting to β-lactam antibiotics should be helpful in drug allergy research and diagnostic tests. We constructed a combinatorial human scFv library and subsequent production of a synthetic IgE with reactivity to amoxicillin and penicillin G. Such synthetic material is used as the standard to develop highly sensitive and reliable *in vitro* allergy tests to harmonize drug allergy testing.

Methods: Phage display was used to generate a synthetic single-chain antibody fragment (scFv) immune library against the major determinants of amoxicillin and penicillin G. The whole IgE molecule was produced by the baculovirus expression vector system and evaluated as a reference material in a chemiluminescence immunoassay. A homologous calibration scheme was built to determine specific IgE to amoxicillin in a cohort of 150 serum samples.

Results: The synthetic IgE resulted in a reliable standard to determine specific IgE to amoxicillin at very low levels. The assay showed a detection limit of 0.12 ng/mL (0.05 IU/mL), and the diagnostic sensitivity increased significantly (73%), quadrupling that of the *in vitro* reference ImmunoCap assay.

Conclusions: Synthetic IgE is a constitutive material produced unlimitedly. It performs as a reliable standard and positive control for laboratory proficiency tests or interlaboratory comparison studies. These achievements will contribute to better labeling allergic patients, thus avoiding the drawbacks associated with the incorrect use of antibiotics, such as the evolution of antimicrobial resistance.

Keywords: allergy, β-lactam antibiotics, diagnostics, recombinant IgE, standard.

INTRODUCTION

The oral ingestion of penicillins is the commonest cause of immune-mediated drug reactions.[1] Indeed, up to 10% of the general population reports being allergic to some β-lactam antibiotics. However, a high percentage of individuals would be classified as tolerant upon accurate clinical and analytical assessment. This mislabeling results in prescribing of alternative antibiotics, triggering other problems such as antibiotic resistance.[2]

Delabeling initiatives are now part of antibiotic stewardship programs in clinical practice worldwide.[3] These actions include risky and time-consuming *in vivo* and low sensitive *in vitro* tests (ca. 81% false negatives), limiting their routine diagnostic use for delabeling purposes.

The growing demand for new *in vitro* diagnostic assays has led to the development of several alternative testing methods based on the immunodetection of allergen-specific IgE (sIgE).[4] Although the assays are based on antigen-antibody recognition, they differ in carrier molecule, antigen, solid phase, sample volume, degree of automation, signal detection, and other parameters.

The diagnostic methods are validated internationally by laboratory proficiency tests or interlaboratory comparison, where the performance data (sensitivity, specificity, accuracy, precision, etc.) are determined.[5,6] Serum samples from allergic patients are required to study the performance of the analytical methods, which is very difficult in the case of allergies to β-lactam antibiotics since the frequently found concentrations of sIgE are low (< 3.5 IU/mL). This deficiency is one of the reasons why, to date, European legislation does not require validation with sera from patients before marketing.[7]

Without a validated method, ImmunoCAP is employed as the reference method. However, the published data reveal that the results of the several *in vitro* tests are not comparable.[8] These discrepancies are due, among other main reasons, to the lack of well-defined quality controls and specific standards to determine immunoglobulins.

The 3rd international standard for serum IgE coded 11/234 is currently used to calibrate assays for serum IgE following a heterologous calibration scheme.[9] The continued availability of a reliable international standard is essential since the exhaustion of stocks necessitates the production of replacement preparations. These preparations are finite and require extensive evaluation in an international collaborative study to determine their suitability.[10] Besides, sera and defibrinated plasma with elevated IgE levels are needed to prepare such reference material.

The use of human sera is the first choice as a control for diagnostic applications, but the total IgE concentration in serum usually is low.[11] Furthermore, sera are severely limited in availability, variable quality, and high cost. The production of synthetic standards could be an exciting alternative to collecting serum and plasma with high IgE levels.

The first attempt to approach synthetic standards consisted of raising specific antibody molecules by hyperimmunization to produce a fully-humanized chimera.[12] However, the generation of these molecules was not fully reproducible.[13,14] A recently reported approach uses nanobodies as standards, but there is still an immunization procedure behind their production.[15,16]

Advances in molecular biology and antibody engineering have made the generation of complete antibodies from single-chain variable fragment antibodies (scFv) libraries possible.[17] The scFv are chimeric proteins produced by fusing the V_H variable regions and the V_L regions of immunoglobulins. In this research line, we report the production of a whole human IgE molecule with defined specificity for amoxicillin and penicillin G. From a human immune synthetic library[18], we selected scFv against the major determinants and established its expression as a human IgE antibody. Expression of the complete human IgE with the desired specificity was performed by the Sf9 baculovirus expression system.[19] This study is the first example of generating such specific human IgE for amoxicillin and penicillin allergy testing produced using a human antibody library and assessed for its biochemical properties by chemiluminescence immunoassay. This

synthetic material was successfully used as the calibrator or standard required for a homologous calibration scheme to analyze a cohort of 150 human serum samples.

MATERIAL AND METHODS

Reagents, buffers, and consumables

Histone H1, penicillin G (PG), amoxicillin (AMX), cefaclor (CFC), isopropyl β-D-1-thio-galactopyranoside (IPTG), polyethylene glycol 8,000 (PEG), 2xTY Medium, Tween 20, DNA T4 Ligase, *Sfi*I, *Mva*I, and other chemicals were from Sigma-Aldrich (Madrid, Spain). Mouse monoclonal antibody anti-human IgE was from Eurofins Ingenasa S.A. (Madrid, Spain). Goat anti-mouse IgG (GAM-HRP) and anti-cMyc-HRP were from Abcam (Cambridge, United Kingdom). The enhanced chemiluminescent substrate solution was from Thermo Fisher (Madrid, Spain) and the ELISA plates were from Costar Corporation (Madrid, Spain).

Phage Display

Blood from a human adult diagnosed as allergic to AMX and PG was used as the lymphocyte source. Following the manufacturer's protocol, the lymphocytes were purified from the drawn blood by gradient centrifugation with Histopaque (Sigma-Aldrich). Total RNA was extracted and retrotranscribed to cDNA using the ReverAID Reverse Transcriptase (Thermo Fisher Scientific). Next, the DNA fragments encoding the V_H and V_L of the immunoglobulins were amplified and assembled by PCR, as described previously.[20,21]

The fragments obtained were digested using *Sfi*I, cloned into the pComb3XSS phagemid vector[22] (pComb3XSS was a gift from Carlos Barbas [Addgene plasmid #63890; http://n2t.net/addgene:63890; RRID: Addgene_63890]), and electroporated into XL1-Blue competent cells. After, cells were cultured and superinfected with KM13 helper phage to generate the phage library. Individual phages obtained in the third round of panning were screened in ELISA plates coated with AMX conjugate. Bound phages were detected with an anti-M13-HRP following the protocol described in the supporting information.

Production and characterization of scFv.

As previously described, the ELISA-positive clones were characterized by *Mva*I restriction analysis and fingerprinting.[17] scFv genes were amplified from single *E. coli*

colonies with the primers ompseq (5'- AAGACAGCTATCGCGATTGCAG-3') and dpseq (5'-AGAAGCGTAGTCCGGAACGTC-3') followed by digestion of the PCR products with *Mva*I. Clones were classified according to the fingerprinting patterns and sequenced to obtain the sequence of the scFv.

The clones grown in 1,000 ml of 2xTY with ampicillin (100 μg/mL) were induced with 0.5 mM IPTG for 16 hours at 25 °C under shaking. The cultures were centrifuged and resuspended in PBS containing 20 mM imidazole, DNase I, and lysozyme. Then, the mixture was sonicated, centrifuged, and the supernatant was purified by affinity chromatography using an FPLC ÄKTA system (GE Healthcare) fitted with a His-Trap column (Thermo Fisher Scientific). The imidazole-eluted fractions were dialyzed against PBS, and their protein content was quantified by Bradford's method. The purified scFv was analyzed by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining method, western-blot, and ELISA as described in the supporting information.

Cloning of IgE cDNA into the Baculovirus Expression Vector

The full-length cDNA encoding the human IgE entire heavy and light chains was synthesized and cloned into pFastBac1 by GeneArt Gene Synthesis (ThermoFisher Scientific). *E. coli* DH10Bac cells were transformed and plated onto LB-agar plates containing 20 μg/ml X-gal, 40 μg/ml IPTG, 7 μg/ml gentamicin, 50 μg/ml kanamycin, and 10 μg/ml tetracycline. The white colonies were selected.

Following the Bac-to-Bac Baculovirus Expression System protocol, the recombinant bacmid DNA was isolated from the transformed bacterial cells, and the recombination (cDNA insertion into the genome baculovirus) was confirmed by PCR with M13 forward and reverse primers (5'-CCCAGTCACGACGTTGTAAAACG-3' and 5'-AGCGGATAACAATTTCACACAGG-3', respectively). Five micrograms of the recombinant bacmid DNA were mixed with 8 μL of FuGene HD reagent (Promega) and added to $9 \cdot 10^5$ exponentially growing cells to transfect the Sf9 cells. Recombinant baculoviruses were harvested three days post-transfection. The viral supernatants were stored at 4 °C. The viral stocks were further amplified by infecting a suspension culture of $3 \cdot 10^6$ Sf9 cells/mL. The cells were incubated with the virus in serum-free SFM900II medium with 1% Pluronic F-68 and appropriate antibiotics at 24 °C with orbital shaking (120 rpm). The viruses were harvested at 72 h post-infection and stored at 4 °C.

Expression, purification, and characterization of rsIgE.

A culture (750 mL) of growing Sf9 cells at a density of $2.5 \cdot 10^6$ cells/mL was infected with the recombinant baculovirus under shaking (120 rpm) at 27 °C. After 96 h incubation, cell culture bottles were harvested by centrifugation at 25,000g for 1 h. The supernatant was purified by affinity chromatography using an FPLC ÄKTA system (GE Healthcare) fitted with a HiTrap™ protein L column (Thermo Fisher Scientific). The eluted fractions were dialyzed against PBS. Then, the protein content of the fractions was quantified spectrophotometrically (Abs 280 nm 0.1% = 1.9) and using Bradford's method. The purified rsIgE was analyzed by western blot and ELISA as described in the S.I.

A dose-response chemiluminescence immunoassay (CLIA) (23) was used to assess the suitability of rsIgE as a standard. For this purpose, we compared the linearity response of serial dilutions of a representative serum sample with a known amoxicillin-specific IgE concentration and the rsIgE diluted in serum.

Analysis of serum samples.

The blood samples were extracted and characterized following standardized protocols by Hospital Universitari i Politècnic La Fe (Valencia, Spain). The participant was enrolled after giving written informed consent according to protocols approved by the ethics review board at Hospital Universitari i Politècnic La Fe (registry no. COBIOPHAD). Procedures were followed by the Helsinki Declaration of 1975, as revised in 2008. The patient was diagnosed as allergic to amoxicillin and penicillin G by the procedure described in the European Network of Drug Allergy (ENDA) protocol based on skin testing, *in vitro*, and drug provocation tests.

Serial dilutions of the produced synthetic IgE (rsIgE) in IgE-free serum were used as the calibrators in the CLIA to determine amoxicillin-specific IgE levels in serum samples.

RESULTS

Phage display.

The recombinant scFv monoclonal antibodies were generated using the cDNA template from the total RNA extracted from human lymphocytes. V_L and V_H domains were successfully amplified by PCR, acquiring the expected molecular sizes of ~350bp and ~400bp, respectively. The two domains' repertories were connected using a long linker (GGSSRSSSSGGGGSGGGG) by PCR, resulting in combinatorial scFV

repertories with a molecular size of about 800bp (Figure 4.1). After that, scFv coding sequences were cloned into XL1-Blue, and an scFv library was generated and used to select phage antibodies against the H1-AMX conjugate. Three rounds of panning were performed. The number of eluted phages was determined by platting, increasing from $2 \cdot 10^4$ PFU to $3.6 \cdot 10^6$ PFU in the third round, achieving a 180-fold enrichment. Eventually, 96 phage clones from the last round of panning were randomly picked up for further testing. Individual phage colonies were tested for H1-AMX binding by indirect ELISA, and 9.4 % (9/96) of them had the potential to specifically binding H1-AMX.

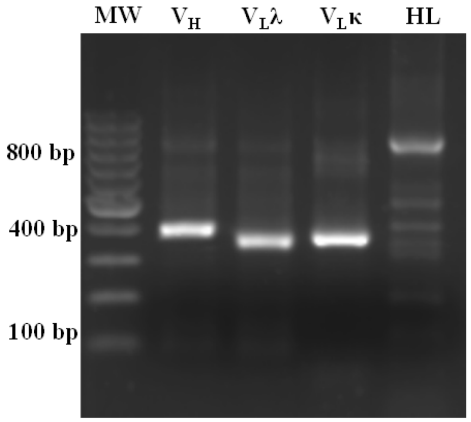

Figure 4.1: PCRs products for amplification of the V_H and V_L ($V_L\lambda$ and $V_L\kappa$) and their assembly into scFv (HL).

Production and characterization of scFv.

The scFv gene of the selected clones was amplified by PCR and subject to *Mva*I restriction analysis. Three different banding patterns were detected (Figure 4.2A). Sequence analysis confirmed that two of them, 1B1 and 1B2, had the V_H and V_L regions, while the 2H6 clone had only the V_L region. The clones with the correct sequence were produced and purified.

1B1 and 1B2 were analyzed by 12 % SDS-PAGE gel electrophoresis and western blot (Figure 4.2B). 1B2 presents a clear and intense band corresponding with an expected molecular weight of around 20 kDa, whereas 1B1 presents a slightly smaller size than expected.

The ability of each clone to recognize the H1-AMX, H1-PG, and H1-CFC conjugates was analyzed by ELISA. As shown in Figure 4.2C, 1B1 was negative since no signal was obtained with the antigens of interest. On the other hand, 1B2 could recognize H1-AMX and H1-PG conjugates, while it did not bind to the H1-CFC conjugate (negative control). H1-CFC is a conjugate formed by the antibiotic cefaclor bound to the same carrier protein

as the other conjugates, confirming this clone's specificity for amoxicillin and penicillin G conjugates. This result reveals that the 1B2 clone selectively recognizes amoxicillin and penicillin conjugate, offering the same reactivity as shown by the patient's clinical history.

Expression, purification and characterizacion of rsIgE

The pFast-Bac1 plasmids containing the light and heavy chain sequences were transformed into DH10-Bac and checked by PCR. As shown in Figure 4.3A, the PCR product obtained from colonies transformed with the light chain plasmid had 3.0 kbp, whereas the heavy chain had 4.0 kbp. In all cases, transformation and transposition were successful, as the product size corresponded to the size of the pFAST-Bac vector (2.3 kbp) plus the insert size.

Recombinant baculoviruses expressing the heavy and light chain of rsIgE were constructed and used to infect Sf9 cells. The purified rsIgE was quantified by Nanodrop and analyzed by SDS-PAGE and Western blotting. Under the experimental conditions, one liter of culture produced one milligram of soluble synthetic IgE. As can be seen in Figure 4.3B, in reducing conditions, two bands appear, one at 20 kDa corresponding to the light chain and another band at 70 kDa corresponding to the heavy chain. In non-reducing conditions, the protein presents a size of 190 kDa, corresponding to the expected mass.

After quantification, the specificity of complete IgE was analyzed by ELISA (Figure 4.3C). The rsIgE explicitly bound to the H1-AMX conjugate and, to a smaller extent, to the H1-PG conjugate, while it did not recognize the H1-CFC conjugate (negative control), keeping the biochemical properties of the scFv used for its construction.

A linearity-of-dilution test was also performed to compare the behavior of rsIgE versus human sIgE in a sample from a patient allergic to amoxicillin. These tests were performed with a reference serum with a known concentration of specific IgE for amoxicillin (3.3 IU/mL as measured by ImmunoCAP), using threefold serial dilutions (1-1/81). Dilutions of the sample and rsIgE were prepared in IgE-free serum. As is shown in Figure 4.4, the linearity was good over a wide range of dilutions, revealing that rsIgE has similar biochemical properties to human sIgE present in serum, demonstrating that the recombinant protein obtained could be used as a standard.

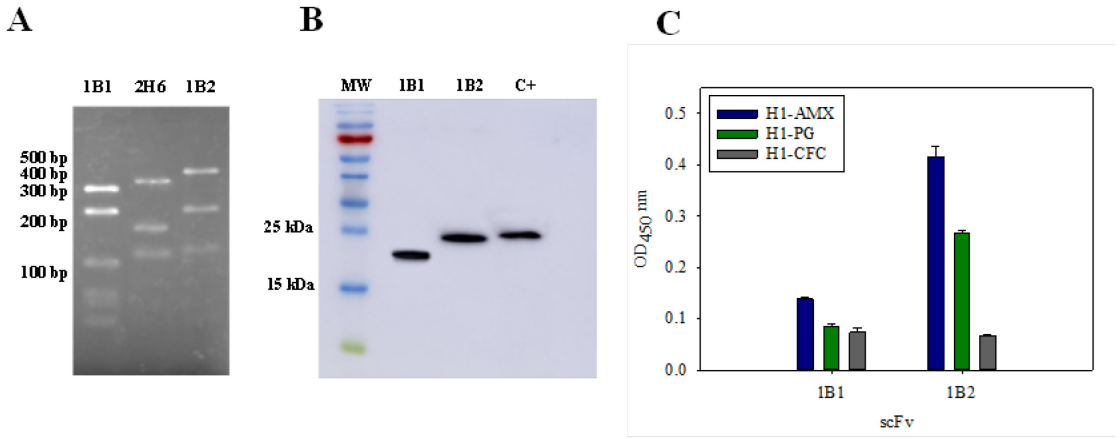

Figure 4.2: [A] *Mva*I fingerprinting analysis of the scFv genes present in the selected clones. The scFv gene inserts were amplified by PCR, digested with *Mva*I and resolved in a 3% agarose gel. Three different banding patterns were detected. [B] Western blot analysis after SDS-PAGE of purified scFv. A previously characterized scFv was used as a positive control. [C] Analysis of scFv antibodies binding specificity by ELISA. Recognition of two conjugates, H1-PG and H1-AMX, by clone 1B2. H1-CFC was the negative control.

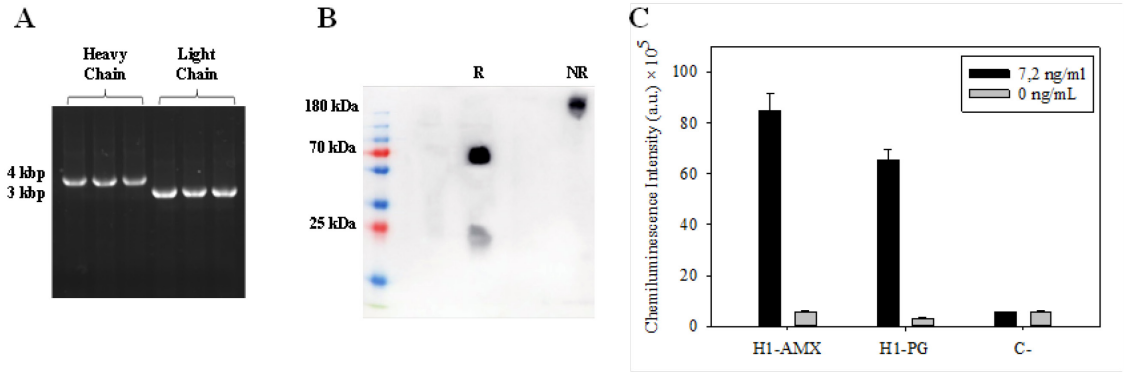

Figure 4.3: [A] PCRs products that confirm the pFast-Bac1 plasmids containing the light and heavy chain. [B] Western blot analysis after SDS-PAGE of purified rsIgE. The rsIgE was analyzed in reducing (R) and non-reducing (NR) conditions. [C] Analysis of rsIgE antibodies binding specificity by ELISA. H1-CFC was used as negative control (C-).

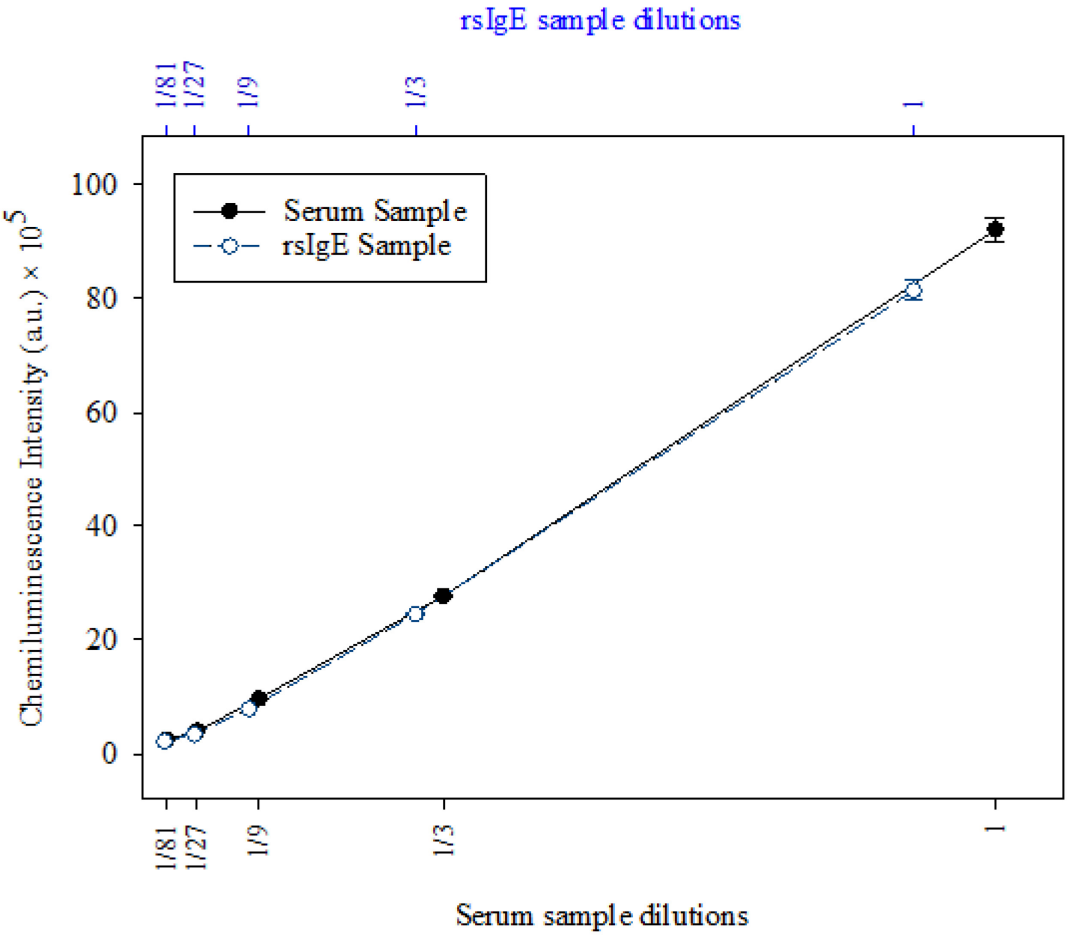

Figure 4.4: Dilution linearity study of a serum sample ($r^2 > 0.99$) and rsIgE ($r^2 > 0.99$).

Analysis of serum samples.

The produced rsIgE was used as a standard to determine the concentration of sIgE to AMX in serum samples, following the homologous calibration scheme in the CLIA method. The current calibration method used to quantify sIgE follows a heterologous scheme and defines 1.0 IU/mL as equivalent to 2.4 ng/mL IgE. In this study, to build the homologous calibration curve, rsIgE was diluted in sIgE-free serum at a concentration of 20, 10, 5, 2.5, 1, 0.75, 0.50, 0.25, and 0.1 IU/mL. Since, in cases of allergy to β-lactam antibiotics, the concentration of sIgE present in serum is not very high, the curve obtained has more points in the lower part so that quantification can be more precise.

The stability of rsIgE in sIgE-free human serum was also studied. The results revealed that there were no statistically significant differences ($p < 0.0001$) between the signals obtained over the studied period (1-8 h). This result implies that rsIgE was stable and compatible with the human serum sample under the assay conditions.

The calibration curve is shown in Figure 4.5. The signals (n=9) were fitted to a four-parameter logistic curve in both cases, giving a dynamic response ranging from 0.1 to 20 IU/mL and a LOD of 0.05 IU/mL in both cases, a concentration below the current internationally accepted cut-off for allergy to β-lactam antibiotic diagnosis (0.35 IU/mL). Regarding the accuracy of the assay, the CVs range from 1 to 10%, resulting in a linear regression with a correlation coefficient (r) of 0.9995. Therefore, the results reveal that the homologous CLIA, using the rsIgE as a calibrator provided excellent analytical performances.

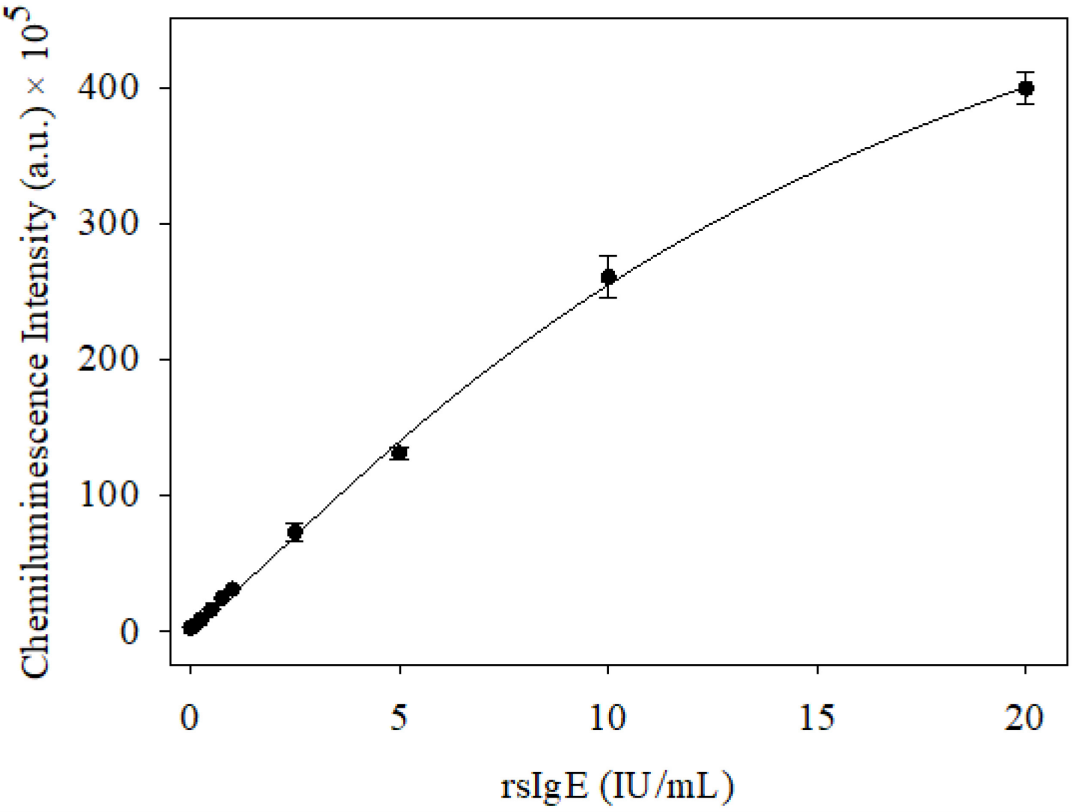

Figure 4.5: Homologous calibration curve.

A cohort of 150 sera collected from 75 patients allergic to amoxicillin, and 75 non-allergic control subjects was analyzed in triplicate by CLIA assay. The signals obtained for each sample were interpolated in the homologous calibration curve. The results, shown in Table 4.1, were compared with the IgE measurements obtained using the current reference system (ImmunoCAP). As observed in Figure 4.6A, the results relate well with those obtained by ImmunoCAP, showing a Pearson correlation close to 1 ($r = 0.999$, $p < 0.0001$). Compared with the reference approach, the results obtained with the homologous

calibration system showed recovery values ranging from 81 to 150%, revealing a reliable quantification.

Table 4.1: Specific IgE to Amoxicillin determined by CLIA and ImmunoCAP (ICAP).

Patient	Culprit Drug	Sex	Age	CLIA (IU/mL)	ICAP (IU/mL)
1	Amoxicillin	F	63	30.30 ± 0.51	28.70
2	Augmentine	M	49	0.30 ± 0.02	0.02
3	Augmentine	F	50	0.40 ± 0.16	0.40
4	Amoxicillin	F	48	< 0.05	0.06
5	Augmentine	F	49	1.10 ± 0.07	0.08
6	Amoxicillin	F	55	0.70 ± 0.02	0.33
7	Augmentine	M	70	1.00 ± 0.07	0.95
8	Amoxicillin	F	38	0.70 ± 0.01	0.07
9	Amoxicillin	F	60	< 0.05	0.00
10	Augmentine	F	34	< 0.05	0.03
11	Amoxicillin	F	69	1.80 ± 0.02	1.65
12	Amoxicillin	F	39	1.40 ± 0.19	0.04
13	Amoxicillin	F	49	0.20 ± 0.01	0.05
14	Amoxicillin	F	68	0.70 ± 0.02	0.65
15	Amoxicillin	F	51	< 0.05	0.04
16	Amoxicillin	M	42	< 0.05	0.02
17	Penicillin	F	66	< 0.05	0.06
18	Amoxicillin	F	46	< 0.05	0.03
19	Augmentine	F	55	0.30 ± 0.01	0.22
20	Augmentine	M	61	0.40 ± 0.01	0.05
21	Augmentine	F	47	< 0.05	0.00
22	Amoxicillin	M	51	1.40 ± 0.01	0.87
23	Amoxicillin	F	68	2.90 ± 0.01	0.08
24	Amoxicillin	F	38	0.20 ± 0.01	0.01
25	Augmentine	M	75	1.40 ± 0.38	0.04
26	Augmentin	F	74	< 0.05	0.00
27	Amoxicillin	F	64	0.30 ± 0.01	0.08
28	Amoxicillin	M	52	0.20 ± 0.01	NR

Patient	Culprit Drug	Sex	Age	CLIA (IU/mL)	ICAP (IU/mL)
29	Amoxicillin	F	57	19.50 ± 0.79	NR
30	Augmentine	M	74	0.50 ± 0.03	0.09
31	Amoxicillin	F	53	0.20 ± 0.01	0.01
32	Augmentine	M	52	9.50 ± 0.05	10.20
33	Cefuroxime	F	77	1.10 ± 0.02	0.09
34	Augmentine	M	71	0.30 ± 0.02	0.02
35	Augmentine	F	59	< 0.05	0.01
36	Augmentine	F	43	7.00 ± 0.01	0.06
37	Augmentine	M	54	0.20 ± 0.01	0.01
38	Augmentine	M	85	0.70 ± 0.06	0.00
39	Augmentine	F	82	0.40 ± 0.03	0.00
40	Amoxicillin	F	59	< 0.05	0.06
41	Augmentine	M	29	< 0.05	0.01
42	Amoxicillin	F	32	0.20 ± 0.03	0.01
43	Augmentine	M	49	0.30 ± 0.02	0.03
44	Augmentine	F	38	0.60 ± 0.09	0.01
45	Augmentine	F	46	0.60 ± 0.07	0.07
46	Amoxicillin	F	58	0.40 ± 0.02	0.04
47	Ceftriaxone	M	32	< 0.05	0.07
48	Augmentine	M	72	0.90 ± 0.05	0.00
49	Augmentine	F	73	1.60 ± 0.08	0.00
50	Amoxicillin	F	54	1.20 ± 0.10	0.00
51	Augmentine	M	50	0.20 ± 0.01	0.01
52	Augmentine	F	61	0.40 ± 0.02	0.03
53	Augmentine	M	65	1.10 ± 0.04	0.95
54	Augmentine	F	39	2.00 ± 0.16	0.00
55	Amoxicillin	M	59	< 0.05	0.05
56	Amoxicillin	F	50	1.30 ± 0.15	0.09
57	Augmentine	M	49	0.20 ± 0.01	0.03
58	Amoxicillin	M	43	0.20 ± 0.01	0.05
59	Augmentine	F	66	< 0.05	0.01
60	Augmentine	F	60	3.80 ± 0.15	0.09

Patient	Culprit Drug	Sex	Age	CLIA (IU/mL)	ICAP (IU/mL)
61	Augmentine	F	43	0.20 ± 0.01	0.01
62	Augmentine	M	57	0.20 ± 0.02	0.08
63	Amoxicillin	M	45	0.40 ± 0.10	0.46
64	Amoxicillin	F	50	< 0.05	0.02
65	Amoxicillin	M	60	< 0.05	0.03
66	Augmentine	M	46	0.50 ± 0.06	0.08
67	Augmentine	M	42	< 0.05	0.03
68	Augmentine	F	32	0.40 ± 0.02	0.09
69	Augmentine	F	57	< 0.05	0.05
70	Amoxicillin	M	53	< 0.05	0.09
71	Amoxicillin	M	39	0.40 ± 0.02	0.04
72	Amoxicillin	M	39	0.80 ± 0.02	0.99
73	Augmentine	F	39	7.00 ± 0.23	7.02
74	Augmentine	F	37	0.40 ± 0.01	0.04
75	Augmentine	F	49	0.20 ± 0.05	0.09

M: male. F: Female.

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 CLIA, 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 CLIA method.

Regarding the sensitivity of the *in vitro* test, the CLIA method identified 55 positives out of 75 (73%), while ImmunoCAP detected 12 positive samples (16%). It is worth mentioning that all the positive results from the ImmunoCAP were also positive by CLIA. Receiver Operating Characteristic (ROC) analysis showed (Figure 4.6B) a good area under the curve for CLIA. Indeed, both *in vitro* tests showed high diagnostic specificity. In summary, the clinical sensitivity of the developed assay was significantly better, quadrupling that of the ImmunoCAP assay, which allowed the identification of more positive allergic patients.

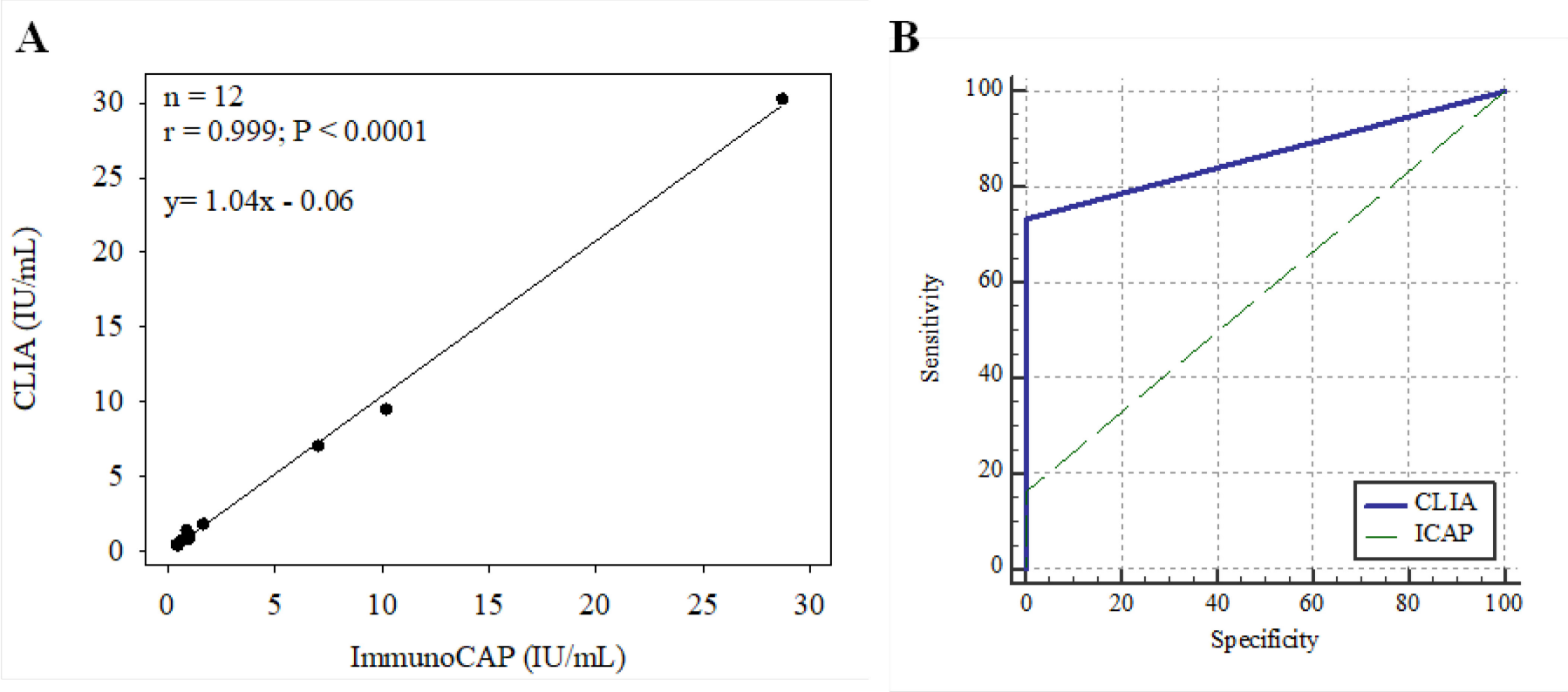

Figure 4.6: [A] Scatter diagram and regression line of inter-method comparison between CLIA with homologous calibration and reference method (ImmunoCAP). [B] ROC analysis representing the area under the curve (AUC). Sensitivity and specificity of the CLIA as compared against ImmunoCAP (n = 150 values).

DISCUSSION

Developing a synthetic and specific IgE is an exciting approach to producing reliable reference standards for *in vitro* allergy testing. In this work, the recombinant-specific IgE obtained specifically recognizes antigenic determinants of amoxicillin and penicillin G. This research demonstrates that rsIgE can be used as a standard for obtaining a homologous calibration curve.

In contrast, using the WHO standard requires bulk material preparation by pooling from more than one source. Each constituent of the bulk material must be characterized and, where possible, identical or meet the essential requirements.[24] The source of this standard is human serum and plasma from allergic patients with high total IgE levels, which implies it is limited. In addition, being a blood-derived product, it requires appropriate regulations since blood is a vehicle for transmitting infectious diseases and emerging agents.[25] These limitations can be overcome with the production of rsIgE. Indeed, the herein presented rsIgE is produced constitutive and unlimitedly with the same biochemical characteristics. These properties make rsIgE a very suitable reference material to determine the reproducibility of a method. Also, its use as a calibrator or standard would minimize inter-method variability to verify whether the new methods produce results that agree with the established procedure, providing the basis for certificates of quantitative analysis on a given material.

On the other hand, rsIgE could be used to assess synthetic antigens used in different *in vitro* tests. Antigenic determinants are the most crucial component of the immunoassay and are responsible for the discrepancies between methods.[26] Therefore, using rsIgE would systematically allow the evaluation of current and future *in vitro* methods. In summary, the work presented here is the first step towards developing specific calibrators to harmonize the standardization of *in vitro* allergy testing methods, which will allow the development of reliable and improved allergy diagnostic tools.

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SUPPORTING INFORMATION

Production of a human synthetic IgE to develop highly sensitive *in vitro* diagnostic testing for β-lactam antibiotic allergy

1. Phage display methodology

1.1. Panning strategy for the selectin of scFv

High-binding plates were coated with 50 µL/well of 10 µg/mL H1-AMX and H1-PG conjugates in a coating buffer (50 mM sodium carbonate/bicarbonate, pH 9.6) at 4 °C for 16 hours. The next day, the wells were blocked with 150 µl TBS 3% BSA for 1 hour at 37 °C, followed by washing with PBS-T 0.05%. The coated wells were incubated with phage particles of the scFv library for 2 h at 37 °C. The wells were washed ten times, and the bound phages were eluted by incubation with 50 µL/well of trypsin at 10 mg/mL for 30 min at 37 °C. Finally, the phages were collected and used for titration and subsequent amplification in *E. coli* XL1-Blue for an additional round of panning. A total of three rounds were performed.

1.2. Screening of positive clones by ELISA

Once the panning rounds were complete, the eluted phages were used to infect *E. coli* XL1-Blue and grown on LB/agar plates. The isolated bacterial colonies were grown in 100 µl 2xTY containing 100 µg/ml ampicillin and 1% glucose in 96 cell-well plates at 37°C overnight.

The following day, in 96 cell-well plates, 200 µl 2xTY containing 100 µg/ml ampicillin and 1% glucose inoculated with 2 µl of single cultures and grown shaking at 37°C for 2 hr. After, 10^9 KM13 helper phage was added and incubated for 1 hour. The cultures were harvested, the pellet resuspended in 200 µl of 2xTY, containing 100 µg/ml ampicillin and 50% kanamycin, and grown by shaking overnight at 30°C. Furthermore, high-binding plates were coated with 100 µL/well of 10 µg/mL H1-AMX and H1-PG conjugates in the coating buffer at 4 °C for 16 hours.

The next day the wells were blocked with 200 µl MPBS 2% for 2 h at room temperature, followed by washings with PBS. 50 µl of each supernatant obtained after centrifugation of the cultures was added to the wells and incubated 1 h at room temperature. Next, the wells were washed with PBS-T, and HRP-anti-M13 (1:5,000) dilution was added and incubated under the same conditions. Finally, after washing,

positive clones were detected by the enzymatic activity developed for 15 min at RT by adding 100 µL/well of HRP substrate (1 mL of 4 mg/mL solution of OPD, 60 µL of 3 % H_2O_2 in water, in a total of 10 mL of 0.1 M acetate buffer, pH 5.5). The enzymatic reaction was stopped by the addition of 50 µL of 1M H_2SO_4, reading the absorbance at 450 nm on a multimode plate reader.

2. Characterization of scFv

2.1. Western Blot

The scFvs were analyzed by SDS-page using 12% gels. The protein was transferred onto nitrocellulose using a semidry system (Biorad) for 13 min at 25 mA. scFv protein was detected by an anti-His monoclonal antibody (1:1,000). HRP labeled anti-mouse antibody was used as the secondary antibody (1:5,000), and the antigen-antibody complexes were visualized by chemiluminescence with ECL (GE Healthcare).

2.2. ELISA assay protocol to evaluate the functionality

An ELISA plate was coated for 16 hours at 4 °C with 5.0 μg/mL of the H1-PG, H1-AMX, and H1-FCF conjugate in a coating buffer. After blocking with PBS-BSA 1% for 30 minutes at 37 °C, the plates were washed four times with PBS-T. Next, 100 μL/well of 10 μg/mL of scFv was dispensed in PBS-BSA 0.1% and incubated for 1 hour at RT with shaking. Subsequently, after washing the plate, 100 μL/well of HRP-c-myc dilution (1:5,000) was dispensed and incubated under the same conditions. Finally, after washing the wells, the result was detected by adding HRP substrate solution.

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3. Characterization of rsIgE

3.1. Western Blot

The rsIgE was analysed by SDS-page using 12% gels. The protein was transferred onto nitrocellulose using a semidry system for 13 min at 25 mA. rsIgE was detected by an anti-human IgE polyclonal antibody (1:1,000). HRP labeled anti-goat antibody was used as the secondary antibody (1:5,000), and the antigen-antibody complexes were visualized by chemiluminescence with ECL (GE Healthcare).

3.2. ELISA assay protocol to evaluate the functionality

An ELISA plate was coated for 16 hours at 4 °C with 3.0 µg/mL of the H1-PG, H1-AMX, and H1-CFC conjugate in a coating buffer. The following day, the plates were washed 4 times with PBS-T. Next, 25 µL/well of rsIgE diluted in sIgE-free human serum were dispensed and incubated for 30 min at room temperature with shaking. Subsequently, after washing the plate, 25 µL/well of monoclonal anti-human IgE solution (0.5 µg/mL) was added to each well and incubated for 15 min at room temperature. Afterward, the ELISA plate was washed as before, and 25 µl of goat anti-mouse IgG solution (2 µg/mL) was added to each well and incubated in the same conditions. Finally, the plate was washed as before, and the peroxidase activity was measured by adding 25 µL of the chemiluminescent substrate solution previously diluted 1/10 in PBS. The luminescent signals were read at 450 nm using a multimode plate reader.

4. CLIA protocol

First, a white flat-bottomed polystyrene ELISA plate was coated with H1-AMX conjugate solutions (3 μg/mL) in the coating buffer (25 μL/well) for the direct determination of specific IgE to amoxicillin. The plates were then sealed and incubated overnight at 4 °C. The next day, the ELISA plate was washed four times with PBS-T, and 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 produced rsIgE diluted in sIgE-free human serum. All serum samples were analyzed in triplicate. Then, after washing the plate, 25 μL/well of monoclonal anti-human IgE solution (0.5 μg/mL) was added to each well and incubated for 15 min at room temperature. Afterward, the ELISA plate was washed as before, and 25 μL of HRP-labelled goat anti-mouse IgG solution (2 μg/mL) was added to each well and incubated in the same conditions. Finally, the plate was washed as before, and the peroxidase activity was measured by adding 25 μL of the chemiluminescent substrate solution, previously diluted 1/10 in PBS. The luminescent signals were read at 450 nm using a multimode plate reader.

5. Results of the controls study

Table S4.1: Results of the analysis of sera from control subjects by CLIA and ImmunoCAP (ICAP).

Patient	Sex	Age	CLIA (IU/mL)	ICAP (IU/mL)
76	F	30	< 0.05	NR
77	F	53	< 0.05	NR
78	M	59	< 0.05	NR
79	F	59	< 0.05	NR
80	M	66	< 0.05	NR
81	F	25	< 0.05	NR
82	M	65	< 0.05	NR
83	F	74	< 0.05	NR
84	F	54	< 0.05	NR
85	F	48	< 0.05	NR
86	F	47	< 0.05	NR
87	F	38	< 0.05	NR
88	F	40	< 0.05	NR
89	F	73	< 0.05	NR
90	F	50	< 0.05	NR
91	F	47	< 0.05	NR
92	M	72	< 0.05	NR
93	F	34	< 0.05	NR
94	F	57	< 0.05	NR
95	F	54	< 0.05	NR
96	M	46	< 0.05	NR
97	F	23	< 0.05	NR
98	F	71	< 0.05	NR
99	F	67	< 0.05	NR
100	F	67	< 0.05	NR
101	F	25	< 0.05	NR
102	F	26	< 0.05	NR
103	F	73	< 0.05	NR
104	F	36	< 0.05	NR
105	F	49	< 0.05	NR
106	F	26	< 0.05	NR
107	F	26	< 0.05	NR
108	M	63	< 0.05	NR
109	F	56	< 0.05	NR
110	F	63	< 0.05	NR
111	F	50	< 0.05	NR

Patient	Sex	Age	CLIA (IU/mL)	ICAP (IU/mL)
112	M	39	< 0.05	NR
113	F	68	< 0.05	NR
114	F	51	< 0.05	NR
115	F	70	< 0.05	NR
116	M	21	< 0.05	0.01
117	F	32	< 0.05	0.01
118	M	55	< 0.05	0.01
119	F	35	< 0.05	0.01
120	F	79	< 0.05	0.01
121	F	71	< 0.05	0.06
122	F	64	< 0.05	0.01
123	F	60	< 0.05	0.01
124	M	82	< 0.05	0.03
125	F	56	< 0.05	0.00
126	M	62	< 0.05	0.05
127	M	63	< 0.05	0.01
128	M	76	< 0.05	0.07
129	M	56	< 0.05	0.03
130	M	46	< 0.05	0.06
131	F	69	< 0.05	0.01
132	F	35	< 0.05	0.00
133	F	42	< 0.05	0.00
134	M	31	< 0.05	0.02
135	F	61	< 0.05	0.00
136	F	71	< 0.05	0.02
137	M	63	< 0.05	0.00
138	F	66	< 0.05	0.01
139	F	49	< 0.05	0.07
140	F	50	< 0.05	0.02
141	F	22	< 0.05	0.00
142	M	68	< 0.05	0.03
143	F	58	< 0.05	0.01
144	F	45	< 0.05	0.02
145	F	72	< 0.05	0.00
146	F	49	< 0.05	0.02
147	F	55	< 0.05	0.02
148	F	49	< 0.05	0.01
149	F	50	< 0.05	0.02
150	F	61	< 0.05	0.01

M: male. F: Female. NR: Not Realized (No evidence of allergy in the clinical history).

5. CONCLUSIONES

CONCLUSIONES

Como resultado de las investigaciones realizadas a lo largo de esta tesis, se ha conseguido llevar a cabo el diseño, obtención y caracterización de proteínas recombinantes con reactividad frente a antibióticos β-lactámicos. Además, se han utilizado dichas proteínas recombinantes para el desarrollo de inmunoensayos *in vitro* para la determinación de sIgE en suero humano con detección luminiscente. Las conclusiones más relevantes con relación a los objetivos particulares propuestos son las siguientes:

1. El inmunoensayo multiparamétrico con detección luminiscente (CLIA) permite la determinación de IgE específica por debajo de 0.1 UI/mL con sólo 25 µl de suero y una hora de ensayo. La sensibilidad clínica es buena (64%) y representa una mejora significativa respecto a la de los métodos en uso para el diagnóstico de la alergia a los antibióticos BLC (25%). En consecuencia, el ensayo CLIA permite diagnosticar la alergia a la penicilina G, la penicilina V, la amoxicilina y la piperacilina con un alto valor predictivo, de forma barata, rápida y sencilla.

2. Se han producido binanoanticuerpos que constituyen una prueba de concepto como calibradores biespecíficos basados en materiales biológicos. La estructura proteica está conformada por dos dominios, uno de ellos capaz de unirse al determinante antigénico de un determinado antibiótico BLC, mientras que el otro puede ser reconocido de manera específica por un anticuerpo anti-IgE. Por lo tanto, los binanoanticuerpos mimetizan el comportamiento funcional de la sIgE a antibióticos BLCs en un inmunoensayo.

3. Los calibradores biespecíficos desarrollados presentan elevada afinidad y selectividad. Además, su extraordinaria estabilidad y su producción de bajo coste por fermentación bacteriana, permite su producción homogénea de forma indefina a partir de su secuencia. Estas ventajas hacen que estas proteínas recombinantes ofrezcan una magnífica oportunidad no sólo para estandarizar las pruebas de alergia, sino también para superar las dificultades de reproducibilidad entre lotes de otros inmunorreactivos.

4. Los binanoanticuerpos biespecíficos se puede utilizar como estándar sintético para la cuantificación de sIgE a antibióticos β-lactámicos en muestras de suero. Esta estrategia triplica la sensibilidad clínica (66%) con respecto al método de referencia (28%) en el diagnóstico de alergia a penicilina G. Esta metodología podría contribuir a la armonización del diagnóstico y facilitar la eliminación de la etiqueta de los pacientes mal categorizados como alérgicos a la penicilina, reduciendo así la propagación de la resistencia a los antibióticos y los costes sanitarios.

5. La IgE específica recombinante (rsIgE) reconoce específicamente los determinantes antigénicos de amoxicilina y penicilina G. Esta se ha utilizado satisfactoriamente como material de referencia fiable para obtener una curva de calibrado homóloga. Los resultados revelan una sensibilidad clínica (73 %) que cuadruplica al método de referencia (16%). Este material se obtiene de forma constitutiva e ilimitada, por lo que se podría utilizar como control positivo en la validación de métodos *in vitro* de determinación de alergias. Además, el uso de estos material fiable y preciso puede ayudar a establecer nuevos reactivos que ayuden a estandarizar los inmunoensayos de diagnóstico de alergia y a minimizar la variación entre ensayos.

En conclusión, se han puesto a punto metodologías de ensayo con fundamentos inmunoquímicos que proporciona información valiosa y complementaria del nivel de sIgE circulante en suero sanguíneo para realizar un diagnóstico *in vitro* de alergia a fármacos más fiable que el de la prueba de referencia. El uso de proteínas recombinantes en este tipo de ensayos permite realizar una calibración homóloga y a estandarizar los métodos en uso. Además, dichas proteínas recombinantes se pueden utilizar para la selección de antígenos de forma sistemática, lo que agilizaría el proceso de obtención de determinantes antigénicos sintéticos pudiendo discriminar aquellos con mejores características analíticas. Todas estas mejoras están asociadas a las directrices marcadas por los planes actuales de uso de antibióticos contribuyendo al correcto etiquetado de los pacientes alérgicos, evitando así los inconvenientes asociados al uso incorrecto de antibióticos, como la evolución de la resistencia a los antimicrobianos.

Además, el uso la metodología Phage Display puede ser empleada para producir proteínas específicas como calibradores y antígenos para todos los antibióticos, lo que conduciría a estrategias terapéuticas personalizadas. Siguiendo en esta línea, las metodologías recombinantes permiten obtener anticuerpos frente, a prácticamente, cualquier diana. Un ejemplo de ello es el nanoanticuerpo obtenido frente a Omalizumab. Este anticuerpo anti-IgE es utilizado en terapia para tratar la urticaria y el asma, y las dosis administradas se basan exclusivamente en los niveles basales de IgE_T y la masa corporal del paciente. El nanoanticuerpo obtenido en esta tesis permitiría el desarrollo de ensayos para monitorizar los niveles de Omalizumab libre en sangre y así poder ajustar la dosis del tratamiento evitando los efectos secundarios asociados a una sobreexposición al fármaco. En definitiva, estas proteínas recombinantes son una herramienta interesante para el desarrollo y mejora de las prestaciones de los métodos diagnóstico no solo de alergias, sino de los tratamientos en inmunoterapia.

CONCLUSIONS

As a result of the research carried out throughout this thesis, it has been possible to design, obtain and characterize recombinant proteins with reactivity against β-lactam antibiotics. In addition, these recombinant proteins have been used for the development of *in vitro* immunoassays for the determination of sIgE in human serum with luminescent detection. The most relevant conclusions concerning the objectives are the following:

1. The multiparametric immunoassay with luminescent detection (CLIA) allows the determination of specific IgE below 0.1 IU/mL with only 25 µL of serum and one hour. The clinical performance is good (64%) and represents a significant improvement over the methods currently used to diagnose allergy to BLC antibiotics (25%). Consequently, CLIA allows diagnosis of allergy to penicillin G, penicillin V, amoxicillin, and piperacillin with a high predictive value, cheaply, quickly, and efficiently.

2. Binanobodies have been produced that constitute a proof-of-concept bispecific calibrators based on biological materials. The protein structure consists of two domains, one capable of binding to the antigenic determinant of a given BLC antibiotic, while an anti-IgE antibody can specifically recognize the other. Therefore, the binanobodies obtained mimic the functional behavior of sIgE to BLCs antibiotics in an immunoassay.

3. The bispecific calibrators developed show high affinity and selectivity. In addition, their extraordinary stability and low-cost production by bacterial fermentation allow their homogeneous production indefinitely from their sequence. These advantages make these recombinant proteins an excellent opportunity to improve the standardization of allergy testing and overcome reproducibility difficulties between batches of other immunoreagents.

4. Bispecific binanobodies can be used as a synthetic standard for quantifying sIgE to β-lactam antibiotics in serum samples. This strategy triples the clinical sensitivity (66%) concerning the reference method (28%) in diagnosing penicillin G allergy. This methodology could contribute to diagnostic harmonization and

facilitate delabeling of patients miscategorized as penicillin-allergic, thereby reducing the spread of antibiotic resistance and healthcare costs.

5. Recombinant specific IgE (rsIgE) recognizes the antigenic determinants of amoxicillin and penicillin G. It has been successfully used as a reliable reference material to obtain a homologous calibration curve. The results reveal a four times higher clinical sensitivity (72 %) than the reference method (16 %). This material is obtained in a constitutive and unlimited form and could be used as a positive control in the validation of *in vitro* methods for allergy determination. In addition, using this reliable and accurate material can help establish new reagents that will help standardize diagnostic allergy immunoassays and minimize inter-assay variation.

In conclusion, immunochemically based assay methodologies have been developed that provide valuable and complementary information on the level of circulating sIgE in blood serum for a more reliable *in vitro* diagnosis of drug allergy than the reference test. Recombinant proteins in this assay allow homologous calibration and standardization with other methods. In addition, these recombinant proteins can be used for the selection of antigens in a systematic way, which would speed up the process of obtaining synthetic antigenic determinants, being able to choose those with better analytical characteristics. All these improvements are associated with the guidelines set by the current antibiotic use plans, contributing to the correct labeling of allergic patients, thus avoiding the drawbacks associated with the incorrect use of antibiotics, such as the evolution of antimicrobial resistance.

In addition, the Phage Display methodology can produce specific proteins for all antibiotics, such as calibrators and antigens, leading to personalized therapeutic strategies. Along the same lines, recombinant methods make it possible to obtain antibodies against any target. An example of this is the nanobody anti-Omalizumab. This anti-IgE antibody is used in therapy to treat urticaria and asthma, and the doses administered are based exclusively on IgE_T levels and the patient's weight. The nanobody obtained in this thesis would allow the development of assays to monitor the levels of free Omalizumab in blood and adjust the treatment dose, avoiding the side effects associated with overexposure to the drug. In short, these recombinant proteins are an

interesting tool for developing and improving the performance of diagnostic methods for allergies and immunotherapy treatments.