

RABBIT ANTI-MOUSE SERUM ALBUMIN POLYCLONAL ANTIBODY GENERATION FOR RELEASE TESTING OF MONOCLONAL ANTIBODIES PRODUCED FROM MURINE ASCITES FLUID

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ABSTRACT

Monoclonal antibodies (mAbs) have become essential analytical tools for biomedical and veterinary diagnosis and the analytical release of other biomolecules due to their high specificity and reproducibility. The mAbs generated in mice contain murine serum albumin (MSA) as the main potential contaminant; this molecule interferes in a large number of tests in which these mAbs are required. For this reason, the development of analytical methods to identify and quantify MSA traces plays an important role in the quality of purified mAb. In this study, an anti-MSA polyclonal antibody (pAb) was generated in rabbit with titers of 1:2 700 000 and purified by nProtein A affinity chromatography with purity greater than 90%. The purified antibody was conjugated to horseradish peroxidase (HRP) by the m-periodate method with an optimal working dilution of 1:64 000 in direct ELISA. Two applications were evaluated for the analytical release of mAbs: identity test by Western Blot and direct ELISA, and albumin quantification by sandwich ELISA. The pAb showed a high specificity capable of identifying MSA traces in the formulations evaluated. The sandwich ELISA demonstrated the ability to quantify MSA in 0.49-125 ng/mL range. These assays are suitable for screening multiple samples, allow rigorous analytical release of mAbs, and assess batch-to-batch consistency.

Keywords: mouse serum albumin, polyclonal antibody, detection, ELISA, Western Blot

INTRODUCTION

Ultra-micro Enzyme Linked Immunoabsorbent Assay (UMELISA)-based serological assays are frequently used for diagnostic because of their high detectability and sensitivity as well as low unspecific reactivity (Morales *et al.*, 2004; Romero *et al.*, 2013; Castells *et al.*, 2013; Hernández *et al.*, 2021). Those characteristic are dependent on the specificity and affinity to the antigen, but also on the antibodies purity. Since the report of the mAbs production method by Köhler and Milstein (1975), there has been an increasing effort to solve problems associated with the purity of mAbs and to improve production efficiency (Liu *et al.*, 2010; Chahar *et al.*, 2019). The mAbs generated in mice contain mouse serum albumin (MSA) as the main potential contaminant. Albumin, the most abundant protein in plasma, is a globular unglycosylated serum protein with a molecular weight of 67 kDa that is synthesized by the liver. Albumin regulates colloidal osmotic pressure and transports hydrophobic molecules, such as lipids, hormones, and toxins (Feldhoff *et al.*, 1985). Albumin contaminant in mAb preparations interferes in a large number of tests in which these mAbs are required, it could be as result of its comparatively huge concentration in sera samples but also the molecule is very flexible and may change its conformation easily in order to bind many diverse ligands (Selby, 1999; Majorek *et al.*, 2012; Bujacz, 2012), on the other hand mammalian serum albumin have high sequence identities (72-82%) and similarities (83-88%) relative to human serum albumin (Chruszcz *et al.*, 2013). Its total elimination depends on a good standardized purification process. For this reason, the development of analytical methods to identify and quantify traces of MSA plays an important role in the release of purified mAb samples. The objective of our study was the generation and evaluation of a pAb against mouse serum albumin to be used as an analytical tool for the detection and quantification of MSA in purified mAb.

MATERIAL AND METHODS

Purification of mouse serum albumin

MSA purification was carried out in two chromatographic steps. First, MSA was purified from ascites with 4 mL of Blue Sepharose 6 Fast Flow matrix (Cytiva,

Life-Sciences, USA) packed in a C 10/10 column (Cytiva, Life-Sciences, USA) previously equilibrated with PBS 1X. Elution was performed with PBS 1X 0.5 M NaCl at a constant flow rate (76 cm.h⁻¹). The second step was carried out in PBS 1X in a C10/40 column (Cytiva, Life-Sciences, USA) loaded with 30 mL of Sephadex G-100 (Cytiva, Life-Sciences, USA) matrix operated at constant linear flow rate 6 cm.h⁻¹.

Immunization scheme

Two white New Zealand male rabbits weighing approximately 1.5 kg free from specific pathogens, supplied by the National Center for Laboratory Animal Breeding (CENPALAB), were immunized subcutaneously with 500 µg of purified mouse serum albumin each one. For the first dose, the antigen was emulsified with a 1:1 ratio in complete Freund's adjuvant. Four subsequent immunizations were performed every 28 days; the first two with 250 µg and the rest with 100 µg of antigen emulsified with a 1:1 ratio in incomplete Freund's adjuvant. Blood samples were taken by puncture of the ear vein to sera titration. At the end of the immunization scheme, exsanguination was performed by cardiac puncture. Collected blood was incubated for 30 min at 37°C and then for 30 min at 4°C. The serum was extracted by centrifugation at 600 x g during 20 min (Hitachi, Tokyo, Japan). Animal experiments were carried out in accordance with the legal requirements of the national authorities.

Rabbit antisera titration ELISA

A polystyrene 96-well microtiter plate (Maxisorp, Nunc, USA) was coated for 1 h at 37°C, with 10 µg/mL mouse serum albumin dissolved in carbonate/bicarbonate buffer pH 9.6 (100 µL/well). Plate was blocked with 3% (w/v) bovine serum albumin (BSA) for 1 h at 37°C and washed four times with PBS 1X, 0.05% (w/v) Tween 20, pH 7.4. Then, rabbit serum was diluted in PBS 1X pH 7.4 and added to the plate to be incubated for 1 h at 37°C. A further washing step was performed again and 100 µL of anti-rabbit antibodies conjugated with HRP diluted in PBS 1X pH 7.4 were applied to the plate and incubated for 1 h at 37°C. Finally, plates were washed again under the same conditions and the reaction was developed using a chromogenic substrate solution of 5.5 mg/mL O-phenylenediamine

dihydrochloride (OPD) dissolved in 0.1 M citrate-phosphate buffer, pH 5.0 and 0.015% (v/v) hydrogen peroxide (H₂O₂). The reaction was stopped after 15 min with the addition of 100 µL of 2 M sulfuric acid (H₂SO₄) and the absorbance was immediately read at 492 nm in a microplate reader (Labsystems Multiskan, Helsinki, Finland). Titration was determined as the maximum dilution in which the absorbance was upper 10X standard deviation of negative serum (n=30).

Cross-reactivity of pAb was evaluated by indirect ELISA similar to the one used in rabbit serum titrations but in this case 10 µg/mL of different purified albumin from other animal sources were used on coating step and purified mAb at 10 µg/mL in PBS 1X was used as sample (**mAb PSA.4**: Prostate Specific Antigen mAb, subclass IgG₁ purified by nProtein A affinity chromatography. **mAb HepB.1**: Hepatitis B surface antigen specific mAb, subclass IgG_{2b}, where two affinity purification process are carried out to achieve high purity. **mAb IgMH**: anti-human IgM mAb). The rest of the procedure was the same.

Determination of total protein concentration

The total protein concentration was determined following the procedure described by **Lowry et al., (1951)** using bovine serum albumin as standard material. The standard curve ranged from 10 to 500 µg/mL, and sample absorbance was measured at 620 nm in a microplate reader (Labsystems Multiskan Plus, Helsinki, Finland).

Purification of mouse albumin specific-polyclonal antibody

Rabbit pAb was purified by affinity chromatography in nProtein A Sepharose 4 Fast Flow matrix (Cytiva, Life-Sciences, USA). An XK 26/20 column (Cytiva, Life-Sciences, USA) with 69 mL of bed and a linear flow rate of 50 cm.h⁻¹ was used. The filtered serum was diluted (1:2 ratio) in binding buffer [1.5 M Glycine, 3 M NaCl, pH 8.9]. Antibodies were eluted using 0.1 M citric acid pH 3.0. The elution fraction pH was neutralized with 2 M Tris-HCl. Subsequently, a buffer exchange to 20 mM Tris, 150 mM NaCl pH 7.0 was done in XK 26/40 column (Cytiva, Life-Sciences, USA) packed with 143 mL of Sephadex G-25 (Cytiva, Life-Sciences, USA) matrix operated at a linear flow rate of 130 cm.h⁻¹. Thimerosal was added as a preservative at a final concentration of 0.02 % (v/v).

Purity analysis by SDS-PAGE

The identity pattern and purity were evaluated by SDS-PAGE 12.5% under both reducing and not reducing conditions, by the procedure described by **Laemmli, (1970)**. For image capture and processing were used IrfanView 4.42 and Image J 1.48V, respectively.

Conjugation of the anti-albumin polyclonal antibody to horseradish peroxidase

Conjugation was performed by the periodate method (**Wilson and Nakane, 1978**). Briefly, 1 mL of purified antibody (8 mg/mL) in carbonate buffer 10 mM pH 9.5 was added to the activated enzyme and it was put on the stirrer for 2 h at 25°C. Then, 0.4 mg of sodium borohydride was added to the conjugated solution. Precipitation by ammonium sulfate was carried out and pellet was resuspended in 1.5 volumes of PBS 1X. The conjugated solution was dialyzed in PBS buffer, and bovine serum albumin was added at a concentration of 10 mg/mL. After BSA was completely hydrated, an equal volume of 99% glycerol was added along with thimerosal solution to a final concentration of 0.01% (v/v).

ELISA for MSA concentration estimation

The MSA quantification in purified mAb samples was performed by an ELISA (**Harlow and Lane, 1988**). Plates (Nunc Maxisorp, Roskilde, Denmark) coated with our anti-MSA polyclonal antibody, at 10 µg/mL in carbonate/bicarbonate coating buffer, pH 9.6 and incubated over night at 4°C. Plates were subsequently washed with PBS 1X, 0.05% (w/v) Tween 20 and blocked for 1 h with PBS 1X, 3% (w/v) powder milk. Each sample was then diluted in PBS 1X, 1% (w/v) powder milk 1:100 and added in duplicate to the plate. Then, the purified MSA was used to prepare the standard curve, in a dilution range from 0.49-125 ng/mL. After 1 h of incubation at 37°C, plates were washed four times with PBS 1X - 0.05% (w/v) Tween 20 and the anti-MSA polyclonal antibody conjugated with HRP was added to the plates in PBS 1X - milk 1% (w/v) and incubated for 1 h at 37°C. Plates were washed again under the same conditions and the reaction was developed using a chromogenic substrate solution composed of 5.5 mg/mL O-phenylenediamine dihydrochloride (OPD) dissolved in 0.1 M citrate-phosphate buffer, pH 5.0 and 0.015% hydrogen peroxide (H₂O₂) (v/v). The reaction was stopped at 15 min with the addition of 100 µL of 2 M sulfuric acid (H₂SO₄) and the absorbance was immediately read at 492 nm in a plate reader (Labsystems Multiskan Plus, Helsinki, Finland). The detection limit was calculated based on absorbance data of the zero MSA standard (assay buffer) as mean value plus 3.3 standard deviations and the quantification limit was calculated as the concentration value corresponding to 10-fold the standard deviation of the blank absorbance (zero

concentration of the MSA standard), according to the international validation guidelines FDA and ICH (**ICH, 2005; FDA, 2015**).

Detection of MSA by Western Blot

For Western Blot assay (**Towbin et al., 1979**), proteins were transferred (100 V) for 1 h from the polyacrylamide gel to a PVDF membrane (Roche Diagnostics, Germany). The membrane was blocked with skim milk powder at 5% (w/v) in TBS-Tween 20. MSA in different samples was detected using the anti-MSA polyclonal antibody conjugated HRP diluted 1:20 000. Colorimetric detection was performed with 4-Chloro-1-naphthol and 0.015% (v/v) hydrogen peroxide (H₂O₂) (Sigma-Aldrich, USA).

RESULTS AND DISCUSSION

In the polyclonal antibodies generation process is crucial the specific activity against the desired antigen. This may achieve in almost all cases with a high purity protein to be used in the immunization scheme. For that reason, a high purity murine albumin was needed for rabbit immunizations.

Purification of mouse serum albumin

The MSA used at the immunization scheme was purified from ascites by Blue Sepharose 6 fast flow matrix followed by size exclusion chromatography. Blue Sepharose is the ideal adsorbent for rapid process albumin purification (**Feldhoff et al., 1985**). Figure 1A shows the results of a typical chromatogram of albumin as eluted from this matrix in the PBS buffer where an elution purity superior to 85% was collected (Fig. 1B, Lane 3).

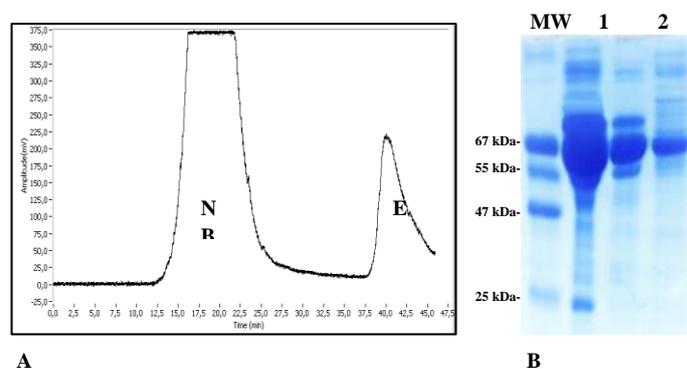


Figure 1 (A) Chromatographic profile and **(B)** 12.5% SDS-PAGE under reducing conditions of Blue Sepharose 6 Fast Flow purification of mouse albumin. MW: molecular weight pattern. Lane 1: ascites, Lane 2: non bound fraction (NB), Lane 3: mouse albumin elution (E).

Molecular exclusion chromatography on Sephadex G-100 succeeded in dividing the albumin into two fractions. The elution pattern presented in Figure 2A is similar to that obtained by **Maclaren et al., (1976)** in his study of mouse albumin aggregates on Sephadex G-150 where the peak preceding the monomer could be shown by ultracentrifugation to be the albumin dimer and earlier peaks, higher polymers of albumin. For the immunization process of the rabbits, the fraction belonging to the tail of the monomer was selected because it had 95% purity (Fig. 2B). The high purity of the antigen in the immunization is needed to avoid antibodies with cross-reactivity against contaminants contained within the antigen solution (**Harlow and Lane, 1979**).

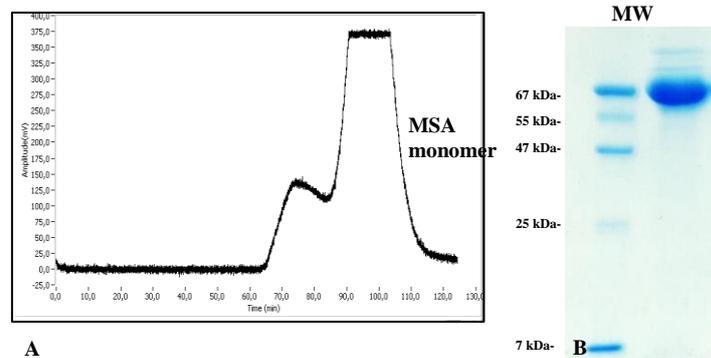


Figure 2 (A) Chromatographic profile and **(B)** 12.5% SDS-PAGE under reducing conditions of mouse albumin purification by size exclusion chromatography on Sephadex G-100. MW: molecular weight pattern. 1: MSA monomer.

Rabbit sera titration and purification of anti-MSA polyclonal antibody

In mAb production for diagnostic purpose and analytical release of other biomolecules, is important to achieve high purity at the purification process to avoid unexpected results in many applications. Sera titration was assessed by direct ELISA and reaction was observed even at 1:2 700 000 dilution factor at the rabbit 1 and at 1:2 301 333 for rabbit 2, respectively (Fig. 3A). This result shows a high specificity of the pAb as well as great MSA-immunogenic properties in rabbits. At the pAb purification throughout nProtein A affinity chromatography was observed just a symmetric peak corresponding to the eluted fraction (Fig. 3B). This affinity purification is the golden standard for antibody preparation, and a technology that has gained high interest because of its great performance and capabilities (Hahn et al., 2006). The SDS-PAGE under reducing conditions showed a reinforced double band at approximately 55 kDa and 25 kDa, with a purity superior to 90% (Fig. 3C, Lane 3). In non-reducing conditions was observed just a homogeneous band of 150 kDa weight with purity higher than 95% (Fig. 3D, Lane 2). The Sephadex G-25 matrix warranted a buffer exchange with a sample recovery superior to 99% (Fig. 3C, Lane 4). The total amount of pAb was 89 mg, achieving a throughput of 8.9 mg/mL serum, superior to others previously reported with similar purification protocols (Hernández et al., 2013; Fernández et al., 2019).

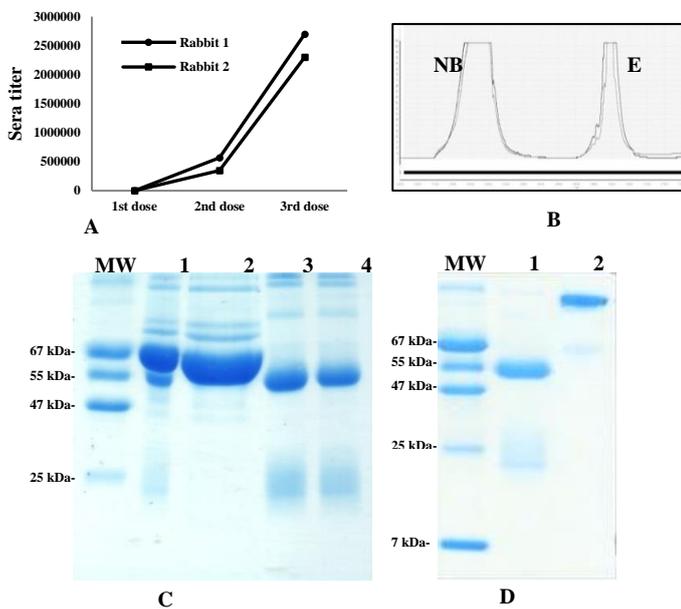
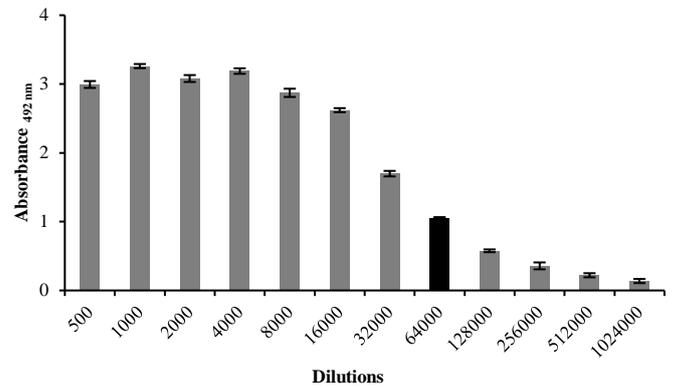


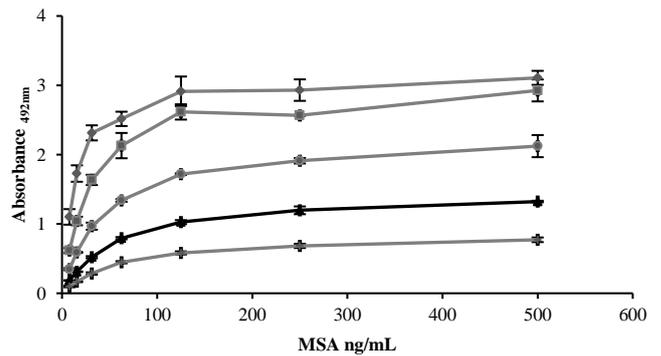
Figure 3 (A) Sera titer of immunized rabbits. (B) Chromatographic profile of nProtein A affinity purification of rabbit anti-MSA polyclonal antibody, NB: non bound fraction, E: antibody elution. (C) 12.5% SDS-PAGE under reducing conditions MW: molecular weight. Lane 1: rabbit anti-MSA serum, Lane 2: non bound fraction, Lane 3: antibody eluted at pH 3.0, Lane 4: antibody buffer exchange throughout Sephadex G-25 matrix and (D) 12.5% SDS-PAGE MW: molecular weight, Lane 1: antibody eluted at pH 3.0 under reducing conditions and Lane 2: antibody eluted at pH 3.0 under non-reducing conditions.

Conjugation of the anti-MSA polyclonal antibody to horseradish peroxidase

For the immunoenzymatic detection of MSA traces in samples of purified mAb, the antibody was conjugated to peroxidase to obtain a biomolecule (pAb-HRP) capable of both to recognize the antigen and to catalyze the reaction between peroxide and substrate used to the colorimetric detection. It is used to avoid additional steps on the immunoassays. The pAb-HRP working dilution was assessed by direct ELISA with coated MSA (10 µg/mL) (Fig. 4A) as well as in sandwich ELISA (Fig. 4B). In the direct ELISA, the pAb-HRP was added at initial dilution of 1:500 and serial dilutions were made 1:2. The optimal working dilution was taken when the observed absorbance was approximately 1 because it would avoid over-saturation of the signal in the plate reader. As shown in Figure 4A, the absorbance was approximately 1 at a 1:64 000 dilution, so this could be taken as a working dilution in direct ELISA. On another hand, in sandwich ELISA, was assessed both the pAb-HRP as well as MSA concentration. The working dilution for this assay was 1:40 000 due to it was approximately 1 in an extensive range. To ensure a better performance of the pAb-HRP in each technique, an optimal working dilution of 1:20 000 was determined for the Western Blot and 1:40 000 for the sandwich ELISA.



A



B

Figure 4 Evaluation of the pAb-HRP in Direct ELISA (A) and sandwich ELISA (B) to determine optimal working dilutions at each immunoassay. (♦): pAb diluted 1: 5 000, (■): pAb diluted 1: 10 000, (●): pAb diluted 1:20 000, (▲): pAb diluted 1:40 000, (+): pAb diluted 1:80 000. In dark, the optimal working dilution for each assay. Values represent the mean of absorbance ± standard deviations (n=3).

Specificity and cross-reactivity of the anti-MSA polyclonal antibody

To determine pAb specificity, is needed to discard cross-reactivity with non-related proteins. Due to the antibody was generated with mouse serum albumin purified from ascites, is important to evaluate reactivity on biological samples to set its potential use to the antigen detection. Cross-reactivity of anti-MSA pAb was assessed in ELISA and Western Blot.

Six possible positive samples were evaluated by direct ELISA to detect MSA and both bovine serum albumin (BSA) and human serum albumin (HSA) proteins. The ELISA results showed that traces of MSA were specifically recognized by the polyclonal anti-MSA antibody to different degrees demonstrating that different amounts of MSA are co-eluted in some mAb lots. On the other hand, there was not cross-reactivity neither with BSA nor with HSA (Fig. 5).

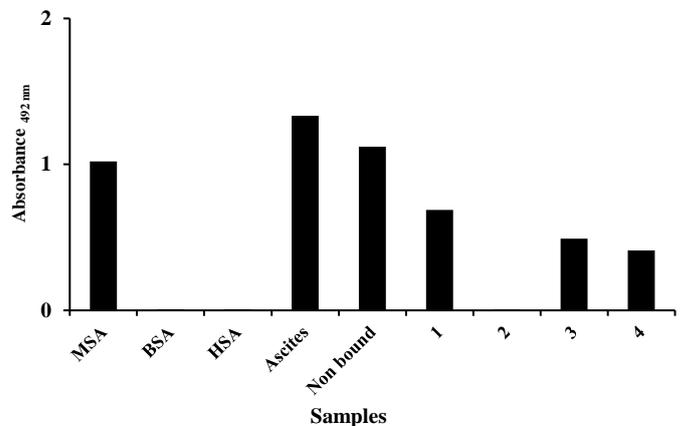


Figure 5 Further study of the MSA traces in different sample of mAb evaluated by direct ELISA. MSA: purified mouse serum albumin was used as positive control, BSA: bovine serum albumin, HSA: human serum albumin, ascites: at the mAb PSA.4, NB: non bound fraction at the mAb PSA.4 purification process, Lane 1: Eluate fraction 1 at the mAb PSA.4 obtained from nProtein A affinity purification process, Lane 2: Eluate fraction 2 at the mAb PSA.4 purification process, Lane 3: Eluted fraction at the mAb HepB.1 (First purification process), Lane 4: Eluted fraction at the mAb HepB.1 second purification process (polishing step).

To confirm the results observed in the ELISA, was performed a Western Blot. A single band at about 67 kDa was detected in every positive sample but was not detected reactivity against murine antibodies neither other proteins contained in the ascites (Fig. 6). This result showed efficiency of immunization and specificity of the pAb to MSA. Although we expected some cross-reactivity against albumin from other animals (due to the homology of this protein among different organisms), the antibody was not capable of recognizing BSA or HSA neither in the direct ELISA nor in Western Blot, at least at the conditions performed in each immunoassay. These results are according to previous works, where anti-albumin pAb and mAb with no cross-reactivity with human and bovine serum albumin have been obtained (Chen et al., 2004; Zhang et al., 2010).

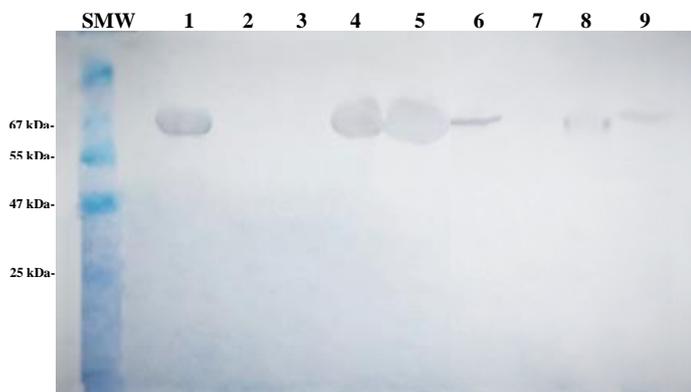


Figure 6 Western Blot performed to assess specificity of the polyclonal antibody with the mouse serum albumin. SMW: Pre-stained SDS-PAGE-Biorad standard molecular weight, Lane 1: purified mouse serum albumin was used as positive control, Lane 2: BSA, bovine serum albumin, Lane 3: HSA, human serum albumin, Lane 4: ascites, Lane 5: non bound fraction at the mAb PSA.4 purification process, Lane 6: Eluate fraction 1 at the mAb PSA.4 obtained from nProtein A affinity purification process, Lane 7: Eluate fraction 2 at the mAb PSA.4 purification process, Lane 8: Eluted fraction at the mAb HepB.1 (First purification process), Lane 9: Eluted fraction at the mAb HepB.1 second purification process (polishing step).

In both immunoassays were evaluated different purification steps of PSA.4 mAb preparation in order to determine albumin traces and we confirmed that it just was successfully removed at the Eluate fraction 2 (Fig 5, Lane 2; Fig. 6, Lane 7). This evaluation was also carried out at the non-conforming mAb HepB.1 purification process, where two chromatographic steps are performed. The immunoassays showed the presence of MSA traces in both purifications, demonstrating that the pAb is a useful tool to the analytical release of mAb preparations.

Determination of the mouse albumin concentration

A sandwich ELISA was performed to assess the potential use of our pAb to quantify MSA traces in purified mAb samples. The method used here was similar to previous works (Fernández et al., 2019; Veitia et al., 2020; Machín et al., 2021) where authors estimated specific protein concentration. From all mAb preparations, the HepB.1 was the higher murine-contaminated (17.8 ng/mL) which correspond with the fact that this mAb product lot was declared non-conforming for quality control. The immunoassay showed to be capable to quantify MSA in a range from 0.49-125 ng/mL. This result is similar with many currently used commercial ELISA (Mouse Albumin ELISA Kit Cat. ab108792 from Abcam corp., Mouse Albumin ELISA Kit Cat. MOA19-K01 from Eagle Bioscience). The sandwich ELISA assay was designed to provide a simple and highly sensitive method to detect traces of contaminating albumin in purified monoclonal antibodies. The MSA detection limit was satisfactory, acceptable for the requirements of most mAb diagnostics. As such, this pAb could be used as a tool to aid in the development of an optimal purification process and in routine quality control during mAb production. The detectability and albumin-specific recognizing of the obtained antibody show suitability for its use to validate a quantification ELISA with the aim of give more accurate results and use it with other purposes like to measure albumin in mouse urine and plasma samples to study various murine diseases in clinical practice.

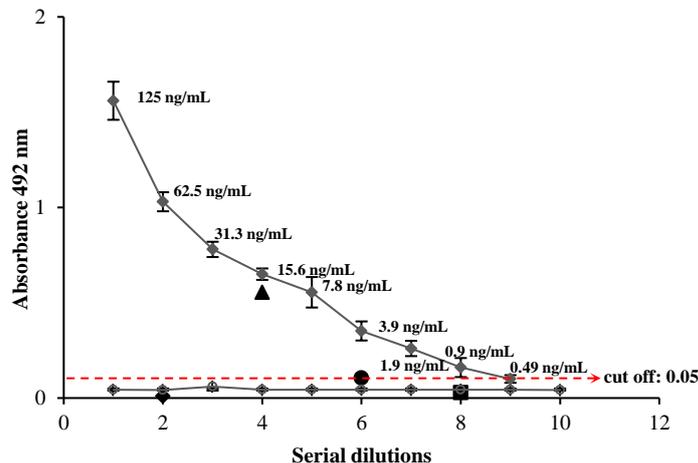


Figure 7 Sandwich ELISA to estimate MSA concentrations in mAb final preparations. In this case, serial dilutions (1:2) of MSA were performed starting from 125 ng/ mL. (◆): PSA.4 final product, (▲): HepB.1 final product (●): IgMH final product, (■): HepBNatural final product (○): recombinant N protein from SARS-CoV2 (unrelated protein).

CONCLUSION

In this study, we report the generation and evaluation of a sensitive and specific polyclonal antibody against mouse serum albumin. High avidity antisera were obtained, which are functional in two different assays, ELISA and Western Blot. The pAb that were purified by nProtein A Sepharose showed that the antibody is highly specific to recognize mouse albumin traces in mAb preparations and hence it could be used at a quantitation ELISA for analytical release of many biological reagents obtained from mouse ascites fluid.

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