Journal of Immunological Methods 471 (2019) 1–10

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Purification of IgG against ribonucleoprotein by a homemade immunoaffinity chromatography column for rabies diagnosis

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ARTICLE INFO

Keywords:
IgG antibody
Rabies
Immunoaffinity chromatography
Direct fluorescent antibody test

ABSTRACT

Polyclonal or monoclonal antibodies against rabies virus ribonucleoprotein (RNP) conjugated to fluorescein isothiocyanate (FITC) have been employed for *Rabies virus* (RABV) antigen detection by the direct fluorescent antibody test (DFA). To date, these biomolecules have been purified by traditional methods such as precipitation by ammonium sulfate or ion exchange chromatography followed by ammonium sulfate precipitation, which allows only for partial detection of the protein of interest. In this study, we aimed to purify anti-RNP polyclonal horse IgG antibodies by cation-exchange chromatography in combination with a homemade immunoaffinity chromatography on RNP immobilized (RNP-IAC). Furthermore, to evaluate the accuracy of the prepared anti-RNP IgG fluorescent antibody in diagnostic purposes, DFA was applied for RABV antigen detection in suspected brain samples of different animal species. The combination of these two techniques made it possible to obtain antibodies with high selectivity and purity. Compared with the performance of the traditional method, anti-RNP IgG antibodies purified by RNP-IAC can be obtained from a smaller volume of hyperimmune serum and with greater avidity. Furthermore, the results obtained by DFA analyses revealed that the prepared anti-RNP IgG fluorescent antibody achieved 100% diagnostic specificity and sensitivity for RABV antigen detection. Thus, two-technique chromatographic, including RNP-IAC technology could be appropriate methods for the purification of polyclonal anti-RNP IgG for the use as a diagnostic reagent for rabies.

1. Introduction

Antibodies are important for basic research and biotechnological processes, highlighting their application as a biological recognition reagent for diagnostic, immunotherapeutic and immunoprophylactic use (Matysiak-Klose et al., 2018; Weisser and Hall, 2009).

In this context, polyclonal or monoclonal antibodies against *Rabies virus* ribonucleoprotein (RNP), conjugated to fluorescein isothiocyanate (FITC), have been employed for *Rabies virus* (RABV) antigen detection by the direct fluorescent antibody test (DFA). The DFA test is considered the gold standard for laboratory diagnosis of rabies and is carried out using central nervous system (CNS) samples of suspected animals or humans. Thus, the performance of this technique depends mainly on the quality of the anti-RABV fluorescent antibody (Goldwasser and Kissling, 1958; Dean and Abelseth, 1996; Rupprecht and Nagarajan, 2015).

The polyclonal antibodies primarily used for the DFA test have been historically obtained from hyperimmune sera of different animal species, such as rabbits, goats, mice and horses (Caporale et al., 2015; Goldwasser et al., 1959; Sabeta and Ngoepe, 2015; ten Veen and Feltkamp, 1969; Tzianabos et al., 1976).

The use of polyclonal antibodies for rabies diagnostic application requires that the antibody be in a purified form. Traditionally, anti-RABV antibody is purified from hyperimmune serum by the ammonium sulfate precipitation method or the anion exchange chromatography associated with precipitation with ammonium sulfate method, followed by the preparation of fluorescent antibody (Caporale et al., 2009; Trimarchi and Debbie, 1974). However, the basis for separation using ammonium sulfate is the preferential precipitation method for IgG at high salt concentration, typically in the range 1.6–2.0 M, and desalting by extensive dialysis is required prior to subsequent analysis. At the lower salt concentration with this range, the precipitate is primarily

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https://doi.org/10.1016/j.jim.2019.03.007
Received 17 December 2018; Received in revised form 8 March 2019; Accepted 19 March 2019
Available online 20 March 2019

composed of IgG, but a substantial amount of the antibody still remains in solution and therefore the recovery is very low (Wang et al., 2008). Additionally, the purification methodology based on ion exchange relies on the separation of biomolecules according to their net surface charge and is commonly used as a polishing step subsequent to Protein-A affinity chromatography (Murphy et al., 2016).

Many methods have been developed for the purification of antibodies from different sources. However, affinity chromatography on immobilized antigens (IAC- chromatography based on antigen–antibody interaction) is often used to isolate specific polyclonal antibodies from a serum (Muronetz and Korpela, 2003; Moser and Hage, 2010; Sheng and Kong, 2012).

Here, we used cation-exchange chromatography associated with a homemade immunoaffinity column for the purification of polyclonal anti-RNP IgG antibodies from hyperimmune horse serum to improve the affinity of the antibody compared to traditional methods. We also evaluated the accuracy of the prepared fluorescent antibody for rabies diagnosis purposes using the DFA test in CNS samples with suspected rabies.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Ethics Committee in Animal Use (CEUA, protocol number 09/2018).

2.2. Virus strains and cells

The virus used in this study was strain CVS-11 (Challenge Virus Standard). The virus was propagated in BHK-21 cells (Baby Hamster Kidney–C-13ATCC CCL-10™), cultivated in Eagle medium (MEM-Vitrocell, São Paulo, Brazil) supplemented with 10% fetal bovine serum (Vitrocell).

2.3. Obtaining rabies virus ribonucleoproteins

2.3.1. Production of RABV in BHK-21 cells

BHK-21 cells were infected with CVS-11 in 30 cell culture bottles of 150 cm^2, as described by Dastkhosh et al., 2014. Two milliliters of BHK-21 cells (10^6/ml) were added to each cell culture flask and incubated at 37 °C until obtaining a 100% confluent monolayer. After 72 h, the medium was discarded and the monolayer of cells was washed with 20 ml of phosphate buffered saline (PBS, GIBCO) solution at a pH 7.4. Subsequently, 9 ml of CVS suspension were inoculated in each flask and incubated for 1 h at 37 °C with 5% CO2. The flasks were gently stirred every 15 min to increase virus adsorption. Subsequently, the cell monolayers were washed with 20 ml of PBS, MEM containing 10% FBS was added, and the volume was adjusted to 50 ml. The bottles were incubated at 37 °C and 5% CO2 for 72 h. After the incubation period, the supernatant was discarded and the cell monolayer was washed with 20 ml PBS.

2.3.2. Extraction of RNP of RABV

Infected BHK-21 cells (item 2.3.1) were collected using a cell scraper (blade length of 38 cm- Midland Scientific TPP) and suspended in ice-cold 0.5 M sodium chloride/Tris-HCl (NT) buffer at pH 7.0 and centrifuged twice at 4 °C and 1000 g for 10 min (Thermo Scientific Sorvall ST 16 R). Cells were lysed by adding 5.0 ml of ice-cold, sterile, deionized water containing 25 μl/ml of aprotinin (Sigma-Aldrich). This mixture was incubated for 1 h at 4 °C and clarified by centrifugation at 1000 *g* at the same temperature for 20 min. The supernatant was collected and this procedure was repeated twice. The supernatants from each round were mixed and centrifuged at 4 °C and 12,000 g for 10 min. Finally, the supernatant was separated for application in the next step.

2.3.3. Purification of RNP

RNP was purified by ultracentrifugation (Hitachi, Himac Gp70β model) in a cesium chloride (CsCl) gradient, according to the protocol described by Dietzschold, 1996. For each 3 ml of the RNP supernatant obtained in the previous step (2.3.2), 2 g CsCl were added to polycarbonate tubes with a volume capacity of 5 ml. The final volume was adjusted to 5 ml by adding NT buffer at pH 7.6. Samples were then centrifuged at 150,000 *g* for 10 h at 4 °C. The band was collected through the top of the tube using a 23-gauge needle and dialyzed against NT buffer with pH 7.6 for 24 h at 4 °C. Subsequently, the protein concentration was determined at a wavelength of 280 nm using a spectrophotometer (Nova Analítica/ Thermo Electron Corporation, BioMate 5). To confirm RNP presence, 5 μL of the purified RNP sample was loaded onto a 12% SDS-PAGE gel with a 5% stacking gel and stained with Coomassie blue (Bio-Rad) according to the protocol described by Laemmli (1970). We also performed a modified Western blot analysis using only the anti-rabies nucleocapsid conjugate (BIO-RAD) to stain a fixed blot of the extracted RNP. A molecular mass standard of known proteins (GE Healthcare) was used as reference.

2.4. Preparation of immunoaffinity chromatography using an immobilized RNP column (RNP-IAC)

2.4.1. Coupling RNP to CNBr-activated Sepharose™ 4B

To prepare the CNBr-activated Sepharose 4B-RNP complex, the coupling procedure recommended by GE Healthacare was followed. RNP was dialyzed into the coupling buffer (0.1 M NaHCO3, 0.5 M NaCl, pH 8.3) overnight at 4 °C. CNBr-activated Sepharose was reswollen in 1 mM HCl at room temperature and filtered. It was then washed twice in coupling buffer. Purified RNP (5.5 mg/ml) was conjugated to cyanogen bromide (CNBr)-activated Sepharose 4B. Sepharose and RNP were mixed end-over-end overnight at 4 °C. RNP-Sepharose was mixed with a blocking agent (0,1 M Tris-HCl buffer, pH = 8.0) for 2 h at 24 °C. Three sets of alternating washes of coupling buffer and acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0) were used to remove non-covalently bound protein from the resin. RNP-Sepharose was stored in ethanol at 20% at 4 °C for further use.

2.4.2. Capacity, recovery and reusability of the IAC column characterization

The polyclonal anti-RNP-antibody binding capacity was determined by the breakthrough volume test (Edinboro and Karnes, 2005). Antiserum solutions of 5.0 ml containing 0.5, 1.0, 1.5, 2.0, 3.0 and 5.0 ml antisera were sequentially loaded on the RNP-IAC. The outlet tubing of the column was connected to an AKTA Prime Plus liquid chromatography system (GE Healthcare). The flow rate was maintained at 0.5 ml/min. The media were washed with 20 mM PBS, pH 7.4 until the baseline was stable. A glycine–HCl elution buffer (0.1 M, pH 2.7, Merck) was used to elute the sample. The anti-RNP antibody solution was collected when the protein peak began to occur. The pH value of the anti-RNP antibody solution was adjusted immediately to neutral with 100 μL of 1 M Tris–HCl, pH 9.0. Quantification of the protein in the elution volume was performed as described above. Column capacity was calculated as follows: column capacity (mg/ml) = maximum bound RNP-IgG/bed volume of column.

To determine recovery, solutions (5.0 ml) containing 0.5, 1.0, 2.0, 4.0, and 5.0 ml antisera were loaded onto the RNP-IAC and HiTrap A-protein High Performance column (5.0 ml, HE Healthacare). The columns were then washed with PBS and eluted with Glycine–HCl buffer. The GIBCO eluate solution during the eluting step was collected, and the protein in the solution was assessed.

The capacity of the immunosorbent to withstand repeated use (reusability) was evaluated with RNP-IAC. The analyte recovery was determined after the column was loaded with 5.0 ml of a solution containing 4.0 ml of antiserum. Subsequently, the columns were washed with PBS until the baseline was stable. Finally, the columns were eluted with a Glycine–HCl buffer. The column was regenerated with 10 ml PBS and reused for a new cycle. The process was repeated > 5 times for RNP-IAC. After each cycle, the concentrations of the protein in the loading, washing, and eluted fractions were measured.

2.5. Extraction of IgG specific antibody from antiserum

2.5.1. Determination of antisera titer

Horse anti-RNP serum was provided by the Instituto Butantan/São Paulo/ Brazil. Immunoreactivity of the serum was checked by indirect ELISA (enzyme-linked immunosorbent assay) according to Nicholson and Prestage (1982) with some modifications. ELISA microplates (Maxisorp, Nun, Roskilde, Denmmark) were coated with 1 μg RNP diluted in 100 μl 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at room temperature. After, the plates were washed four times with PBS containing 0.05% Tween 20 (PBST) and then blocked with 3% bovine serum albumin (BSA, Sigma –Aldrich) for 2 h at room temperature. The plates were washed again four times with PBST and incubated with 100 μl horse antisera against RNP at 8 different dilutions between 1:2000 and 1:512,000 for 1 h at 37 °C. Non-immune serum was used as a negative control. Following the washing step, plates were incubated for 1 h at 37 °C with 100 μl of diluted HRP-conjugated goat anti-horse IgG (1:25,0000, Sigma-Aldrich). The reaction was developed by adding 100 μl TMB (3,3′,5,5′-tetramethybenzidine, Sigma-Aldrich) for 10 min at room temperature. Finally, 50 μl 1 N sulfuric acid were added to each well (Sigma-Aldrich) and the absorbance was determined at 450 nm using a microplate reader (Thermo Scientific Multiskan GO). The antiserum with the highest reactivity was submitted to antibody purification procedures.

2.5.2. Isolation of horse IgG

The IgG was isolated from hyperimmune horse anti-RNP serum by the ion exchange chromatography method on a (QAE, cation exchanger)-sepharose column (GE Healthcare). Samples (equivalent to 25 ml serum previously dialyzed against 50 mM ethylenediamine buffer (TED) at pH 7.2 for 24 h (Sigma-Aldrich)) were applied to a QAE-sepharose column (XK 16 × 40), connected to an AKTA Prime Plus liquid chromatography system (GE Healthcare) and equilibrated with the same buffer. The flow-rate was 1.00 ml min-1. The protein-containing fractions (IgG) eluted with TED were pooled and concentrated (Amicon Ultra-15, 30 KDa),

2.5.3. Purification of specific IgG antibody by RNP-IAC column

Purified total IgG samples were dialyzed in 0.01 M sodium phosphate buffer (NaH2PO4, Merck) pH 7.0. The sample was applied to the RNP-IAC column, which was previously equilibrated in 0.01 M binding buffer (NaH2PO4, pH 7.0), and then 5.0 ml of the same binding buffer was applied. The resin bound anti-RNP antibodies were eluted with a 0.1 M glycine solution (pH 2.7) and the fractions were collected in a volume of 1.0 ml/tube. The fractions containing the anti-RNP IgG were concentrated in a membrane centriprep system (Amicon cut off 30 KDa), dialyzed against 0.01 M PBS, pH 7.2 and the protein concentration was determined by a spectrophotometer at a wavelength of 280 nm (Stoscheck, 1990).

2.6. Characterization of purified anti-RNP IgG antibody

The anti-RNP IgG purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and reducing conditions at neutral pH according to the standard Laemmli protocol (Laemmli, 1970). The concentration of polyacrylamide solution was 10%. Samples were boiled with 2% SDS (Merck) for 5 min and 0.20 μg were loaded for each sample well onto an electrophoresis gel in a vertical chamber. Electrophoresis was performed in a mini-PROTEAN® electrophoresis instrument (Bio-Rad Laboratories, Hercules, USA). After separation, the antibodies were stained with Coomassie Brilliant Blue G 250 (Bio-Rad) and imaged.

In addition, the reactivity of the purified antibody was determined by indirect ELISA. For this, an ELISA microplate (Maxisorp, Nun, Roskilde, Denmmark) was coated with purified RNP (1 μg/100 μL), prepared in duplicate, and incubated overnight at 4 °C. The plates were washed three times with PBS-Tween-20 (PBST: 0.05% Tween-20), and then blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich). After three wash cycles, the plate was incubated with purified anti-RNP IgG antibody for 1 h at 37 °C. Subsequently, anti-IgG horse peroxidase (Sigma-Aldrich), diluted 1:25.000 in 1% BSA, was added and incubated under the same conditions. The reaction was detected by incubation with 100 μL substrate system for ELISA (3,3′,5,5′-tetramethylbenzidine –TMB/Sigma) and colorimetric reactions were stopped by the addition of 1 N sulfuric acid (Sigma-Aldrich). The optical density (OD) of wells was measured at a wavelength of 450 nm using an ELISA Reader (Thermo Scientific Multiskan GO).

The affinity/avidity of antigen-specific antibodies in serum and purified Ig was measured by indirect competitive ELISA using sodium thiocyanate (NaSCN) as previously described (Pullen et al., 1986; Maeda et al., 2017). Briefly, the plates were coated with RNP as described above. The hyperimmune horse serum was tested at dilutions corresponding to an OD450 of 0.8, while purified anti-RNP IgG antibodies were evaluated at an OD450 of 0.5. After incubation with sera or purified antibodies, different concentrations of NaSCN were added to wells and incubated for 15 min. The plates were washed and incubated with HRP-conjugated anti-horse IgG antibody and processed following the standard ELISA protocol. The percentage of antibodies bound to RNP was determined: OD450 in the presence of NaSCN × 100/ OD450 in the absence of NaSCN. The values are expressed as a percentage of antibodies that remain bound to the solid phase-adsorbed antigen under presence of NaSCN in regard to the reaction performed without NaSCN. For purposes of comparison, the anti-RNP IgG antibody sample purified by the traditional method described by Caporale et al., 2009, was used for this assay. The effective concentration of NaSCN required to release 50% of specific serum antibodies (ED_{50}) was determined and used to compare the avidity of the antibodies recovered by the two purification methods.

2.7. Conjugation of anti-RNP IgG antibody with FITC

The anti-RNP IgG purified by both methods was dialyzed against reaction buffer (500 Mm Carbonate, pH = 9.2) for 24 h. One milligram of FITC (Sigma) was dissolved in 1 ml anhydrous dimethyl sulfoxide (Sigma-Aldrich) before use. The FITC was added to give a ratio of 60 μg per 0.50 mg IgG and mixed immediately at 4 °C (overnight). After conjugation, the unreacted FITC was removed by gel filtration on a Sephadex G column equilibrated and eluted with PBS pH 7.0.

2.8. Determination of conjugated anti-RNP IgG reactivity

2.8.1. Titration of the anti-RNP IgG conjugated to FITC

For titrating the conjugate, impressions of the CNS were made from rabies-infected mice, air dried for two minutes at room temperature (22–28 °C), fixed in chilled acetone at 20 °C for an hour, and air dried again at room temperature. Serial two-fold dilutions (1:10 to 1:200) of the conjugate made in PBS pH 7.0 containing 0.01% Evan's blue (Sigma-Aldrich) were applied to the slides and incubated for 30 min at 37 °C in a humidified chamber. After drying, samples were observed in a fluorescence microscope (ZEISS microscopy, HAL-100) with 400× magnification. Each stained slide was observed by three persons independently, and the criterion of the last dilution of the conjugate providing a crisp and high fluorescent staining with minimal background was considered as the end-point dilution.

2.8.2. Determination of diagnostic sensitivity and specificity of anti-RNP IgG conjugated to FITC

A total of 125 CNS samples were used for this evaluation, including:

canine (n = 24), feline (n = 10), bovine (n = 28), equine (n = 18), bat (n = 41) and nonhuman primates (n = 4). All samples had been sent between January and August 2018 and were submitted to the diagnostic laboratory at the Instituto Pasteur- São Paulo, Brazil. For this, three slides of each sample were prepared with brains tissue imprints, using the same fragment always. The slides were fixed with ice-cold acetone for 45 min in a freezer at −20 °C. For each reaction, positive and negative SNC samples were used as controls. The anti-RNP IgG-FITC, diluted at the previously determined titration (item 2.8.1) was added to two slides. Anti-RNP IgG and a commercially available FITC-conjugated anti-rabies monoclonal antibody was used in the study as reaction control. The calculations for sensitivity and specificity of anti-RNP IgG antibody were expressed as equations as described by van Stralen et al., 2009.

2.9. Statistical analyses

The data are represented as the arithmetic means ± SD and analyzed for variance (ANOVA) with a subsequent Bonferroni's multiple comparison test using GraphPad Prism v7 software. For the data generated by the avidity assay, the statistical analyses were calculated from three independent experiments.

3. Results

3.1. Antiserum titer analysis by ELISA

The production of antibodies in the horse and the effectiveness of immunization was verified by an indirect ELISA assay. The antibody titer is defined as the highest dilution of serum at which the OD450 ratio is 2:1. Thus, for the negative control (nonimmune serum) at a dilution of 1/256,000, the OD450 was 0.048 and was 0.388 for the antiserum at the same dilution. Therefore, the ratio is > 2:1, and the RNP antiserum titer was high at ~1/256,000 (Fig. 1A), which indicated that a strong humoral response has been generated, and thus, the specific IgG antibody could be purified.

3.2. Analyses of RNP-IAC column performance

3.2.1. Capacity, recovery and reusability of RNP-IAC column

The amount of target protein that could be absorbed by the column, i.e., its capacity, was evaluated. Fig. 1B. showed the amount of RNP-antibody IgG bound onto the RNP-IAC with the antiserum load. When the loaded antiserum (the dotted curve) was varied from 0.5 to 5.0 ml, the quantity of anti-RNP antibody purified by the RNP-IAC increased linearly until the bound antibody reached the maximum at 11.85 mg. The bed volume of the RNP-IAC was approximately 5.0 ml; thus, the column capacity was 2.37 mg/ml. The recovery of the RNP-IAC is shown in Table 1. The HiTrap protein A column showed the content of total IgG in the polyclonal antiserum, whereas RNP-IAC showed the content of anti-RNP IgG antibody in the polyclonal antiserum. Thus, the amount of anti-RNP IgG accounted for approximately 70% of the total amount of the IgG in the antiserum. In addition, excessive amounts of antiserum (4.0 ml) were continuously loaded onto the RNP-IAC column to test column stability. After 5 repeated uses, no obvious decrease in amount of the antibody purified, relative to that of the purified IgG on

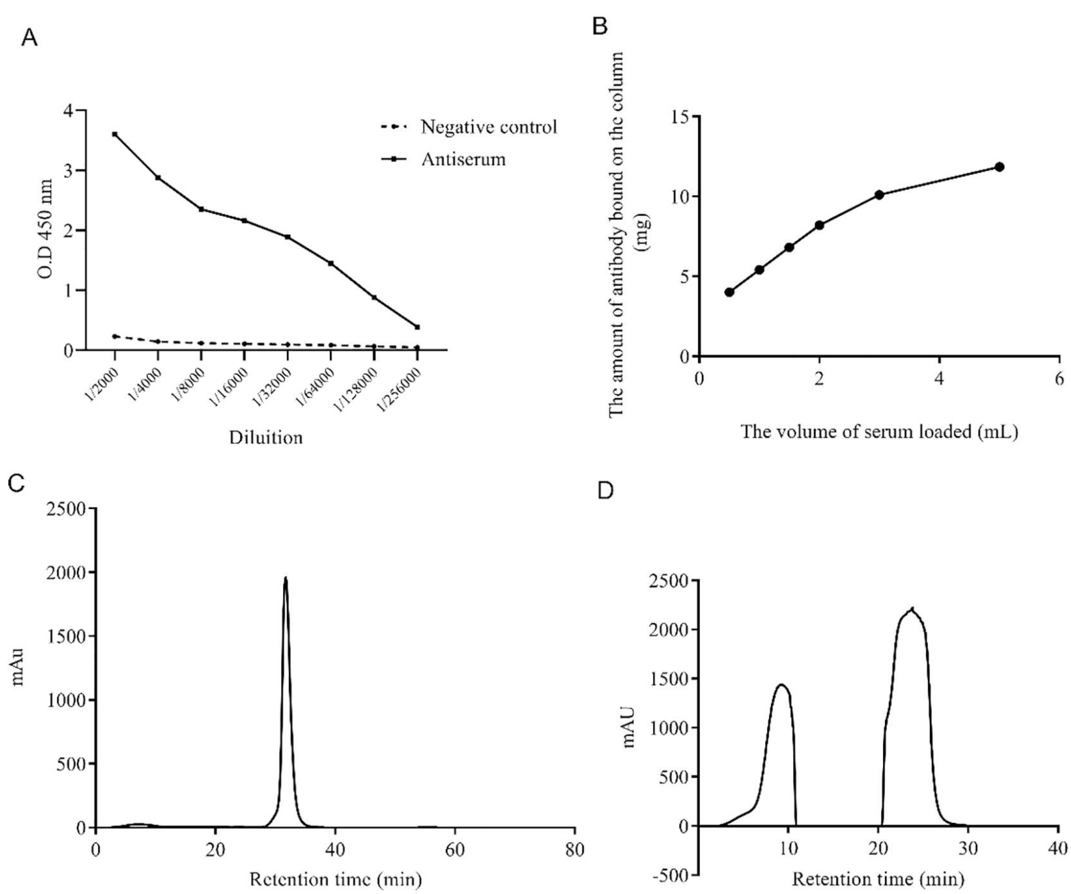

Fig. 1. Obtaining the anti-RNP polyclonal IgG antibody. (A) Analysis of anti-RNP IgG titer in the serum of immunized horse by ELISA. Negative control (nonimmune serum). (B) Amount of antibody bound onto the RNP-IAC (immunoaffinity chromatography on immobilized RNP) in relation to the antiserum. (C) Isolation of horse IgG by ion-exchange chromatography on QAE sepharose. (D) Chromatogram showing the purification profile of anti-RNP polyclonal IgG using a 5 ml RNP-IAC column; the second peak was eluted with 100 mM glycine, pH = 2.7.

Table 1
Recovery of the immunoaffinity chromatography and HiTrap protein A column for anti-RNP IgG antibody.

Antiserum loaded (ml)	HiTrap A (mg/ml)	RNP-IAC (mg/ml)	IAC/HiTrap A (%)
1.0	9.20	6.50	70.65
2.5	14.40	10.65	73.95
4.0	18.70	11.75	62.83
5.0	18.35	11.75	64.03

RNP-IAC = Immunoaffinity chromatography on immobilized RNP.
Hitrap protein A = Affinity chromatography on immobilized Protein A Sepharose.

first usage, was noted. The column capacity decreased to approximately 95% after 7 usages. In fact, the column could be used stably throughout the five cycles tested.

3.3. Purification of horse anti-RNP IgG immunoglobulins

The protein content of the horse serum was 55.82 mg/ml. The purification of polyclonal antibodies from an immunized horse by QAE ion-exchange chromatography (IEC) resulted in a highly pure fraction. The protein content of this fraction after elution from the IEC was 18.3 mg/ml of horse polyclonal IgG, which was about one third of the primary protein content (Fig. 1C).

After the dialysis stages, 45.75 mg of proteins were loaded onto the IAC column. Purification by the immunoaffinity method resulted in approximately 16 mg/ml of horse polyclonal anti-RNP IgG (Fig. 1D).

3.4. Assessment of the purity, reactivity and avidity of the anti-RNP polyclonal IgG

The results of the SDS-PAGE for determining the purity of horse anti-RNP IgG (which were previously purified by ion-exchange chromatography followed by RNP- IAC) are shown in Fig. 2. In the non-reduced SDS-PAGE results, one band with a molecular weight of approximately 225 kDa was present, which demonstrated whole antibody purification (Fig. 2A). The results of the reduced SDS-PAGE for determining the purity of polyclonal horse IgG showed a distinct band with a molecular weight of approximately the 52 kDa position corresponding to the antibody heavy chain, while the bands of molecular weights of 24 kDa indicate that these are the light chains of IgG (Fig. 2B). SDS-PAGE analysis showed that the purification of IgG by the ion-exchange chromatography associated with the RNP-IAC method resulted in a highly pure and acceptable product.

To determine the reactivity of the purified IgG antibodies, an indirect ELISA was performed. An IgG purified dilution (0.625–40 ng/ml) was reacted with an equal amount of RNP. The result indicated that the purified antibody had a high affinity for RNP (Fig. 3A) and a similar reactivity (high reactivity) as that of the original horse antiserum (Fig. 3B).

Furthermore, high avidity was detected in RNP-specific IgG antibodies purified by RNP-IAC compared to antibodies obtained by traditional methods (Fig. 3C). By increasing the volume of the antiserum (120 ml) used in the purification process by the traditional method, samples from three independent purification experiments were tested and no significant difference in the binding avidity (ED_{50} = 2.0 M for both anti-RNP IgG samples) was observed regardless of the purification method (Table 2).

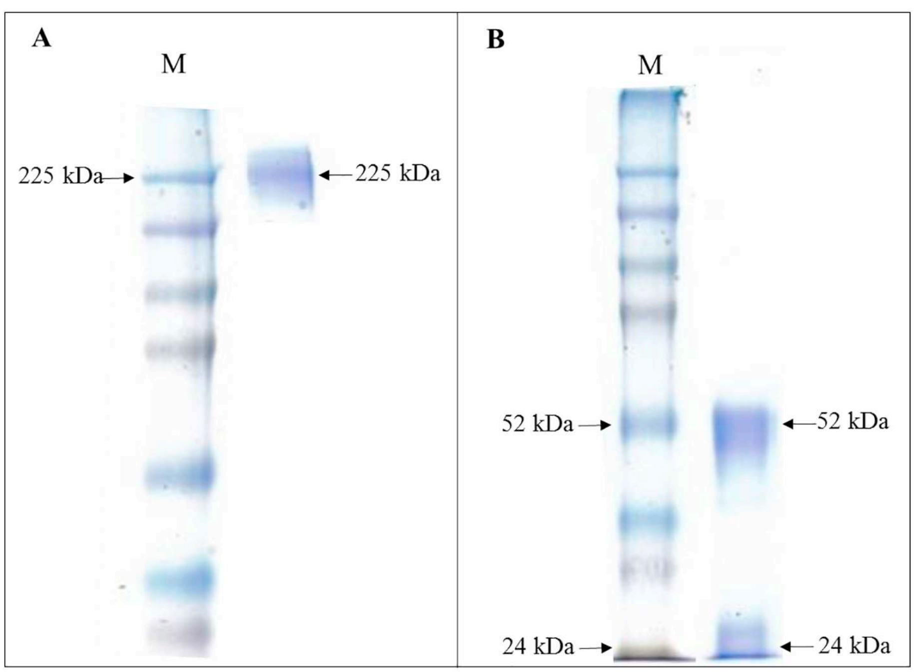

Fig. 2. SDS-PAGE analysis for purity determination of purified horse anti-RNP IgG. A- Nonreducing condition; B- reducing condition. M = Molecular mass standards (Amersham, Cod. RNP800E).

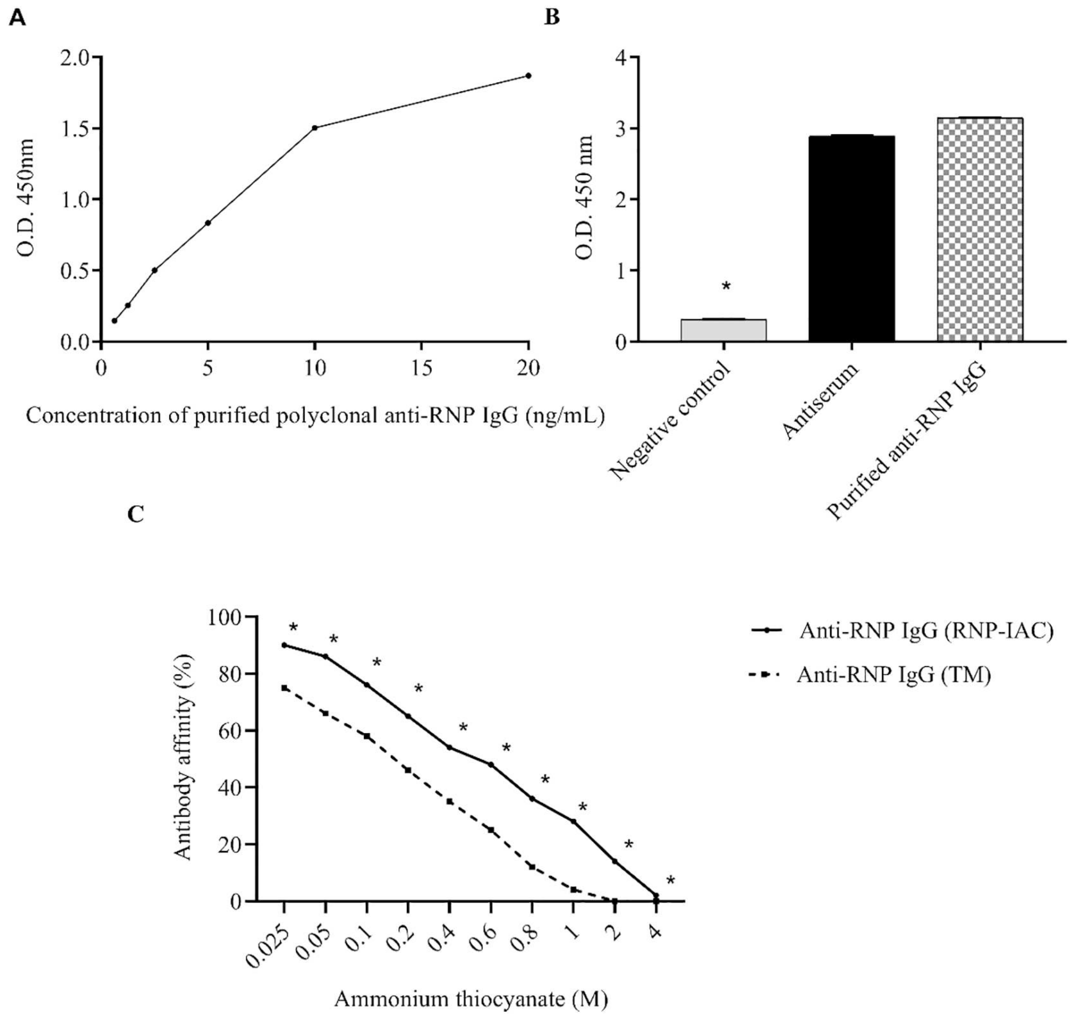

Fig. 3. Characterization of purified anti-RNP IgG antibody. (A) Indirect ELISA for testing the reactivity of the anti-RNP IgG antibody. (B) Purified anti-RNP IgG shows similar reactivity as that of the original antiserum by indirect ELISA assay. * $p < .001$ compared to negative control. (C) High antigen affinity was detected in anti-RNP IgG (RNP-IAC) compared to IgG antibody purified by the traditional method (TM). Values are expressed by percentage of antibodies that remain bound to the solid phase-adsorbed RNP in the presence of ammonium thiocyanate in regard to the reaction performed without ammonium thiocyanate. *p < .001, statistically significant difference. RNP-IAC = Immunoaffinity chromatography on immobilized RNP; TM = Traditional method (ion exchange chromatography followed by ammonium sulfate precipitation method).

Table 2
Comparison of polyclonal IgG against RNP purified by immunoaffinity chromatography and traditional method.

Volume of serum used (ml)	Purification method	Average conc. (mg/ml)	Binding avidity (ED_{50})
25 ml	RNP-IAC	16.0	2.0
25 ml	Traditional	18.1	0.8*
120 ml	Traditional	88.3	2.0

RNP-IAC = Immunoaffinity chromatography on immobilized RNP.
Traditional method = Ion exchange chromatography followed by ammonium sulfate precipitation method.
ED_{50} = The effective concentration of NaSCN required to release 50% of specific serum antibodies.
* p < .001, compared with the ED_{50} obtained by RNP-IAC method.

3.5. Effectiveness of the usage of the anti-RNP IgG in the DFA

The purified polyclonal IgG antibody was conjugated with FITC. The quality control of fluorescent conjugates is usually performed by spectrophotometry, where the ratio between fluorochrome and protein (F/P ratio) is measured. The anti-IgG-FITC had a fluorochrome/IgG protein molar ratio of approximately 2.8 and the optimum dilution of prepared FITC-conjugated anti-RNP IgG was 1:30 in a DFA test.

The ability of the purified anti-RNP IgG antibody to recognize the RABV antigen was analyzed in the CNS samples from animals by an

Table 3
Qualitative results de all samples tested by direct fluorescent antibody test with anti-RNP IgG polyclonal antibody purified by immunoaffinity chromatography and the anti-RNP IgG polyclonal antibody purified by traditional methods.

Species (test number)	Anti-RNP IgG (RNP-IAC)	Anti-RNP IgG (TM)
	Positive Negative	Positive Negative
Canine (*n* = 12)	0 12	0 12
Bovine (*n* = 8)	8 0	8 0
Equine (*n* = 6)	2 4	2 4
Bat (*n* = 11)	7 4	7 4
Feline (n = 1)	0 1	0 1
Monkey (n = 1)	0 1	0 1
Total	17 22	17 22

RNP-IAC = Immunoaffinity chromatography on immobilized RNP.
TM = Traditional method (anion exchange chromatography associated with ammonium sulfate).

immunofluorescence assay. Initially, the DFA test was conducted to determine the specificity and sensitivity characteristics of anti-RNP IgG-FITC purified by RNP-IAC, and using as reference the conjugate obtained by the traditional method (Table 3 and Fig. 4). The analyses of samples by DFA revealed that the conjugated antibody produced obtained 100% of diagnostic specificity and sensitivity for RABV detection, and this was in good agreement with the results obtained with the conjugate antibody used as a reference.

To evaluate the quality of the fluorescence of the reaction, the results of the DFA using anti-RNP IgG-FITC were compared to those obtained using commercial antibody. From the 125 samples analyzed, 51 were positive and 74 were negative by the DFA test, showing agreement between the results obtained regardless of the conjugate used in the test (Table 4). In addition, no difference in fluorescence intensity was observed between the conjugates analyzed, indicating that the anti-RNP IgG-FITC antibody prepared in-house has a quality standard comparable with a commercial reagent (Fig. 5).

Table 4
Qualitative results de all samples tested by direct fluorescent antibody test with anti-RNP IgG polyclonal antibody purified by immunoaffinity chromatography and the commercial reagent.

Species (test number)	Anti-RNP IgG (RNP-IAC)		Commercial reagent	
	Positive	Negative	Positive	Negative
Canine (n = 24)	2	22	2	22
Bovine (*n* = 28)	23	5	23	5
Equine (*n* = 18)	10	8	10	8
Bat (*n* = 41)	16	25	16	25
Feline (n = 10)	0	10	0	10
Nonhuman primates (n = 4)	0	4	0	4
Total	51	74	51	74

RNP-IAC = Immunoaffinity chromatography on immobilized RNP.
Commercial reagent = FITC-conjugate anti-rabies monoclonal antibody/Merck Science.

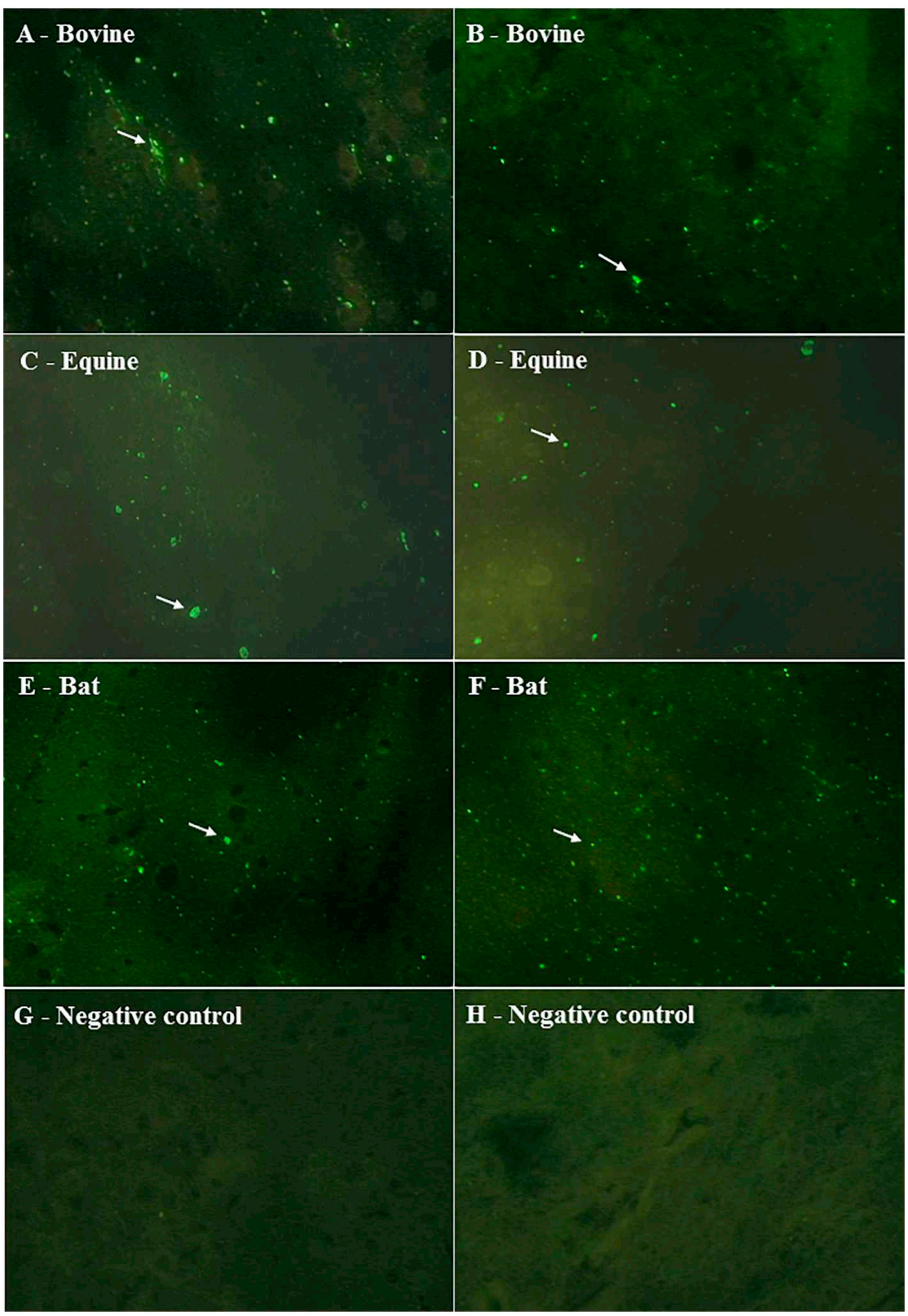

Fig. 4. Representative images of a touch impression of rabies RABV-positive animal brains testing by direct fluorescent-antibody assay. (A, C, E, and G) anti-RNP IgG polyclonal antibody purified by immunoaffinity chromatography on immobilized RNP (RNP-IAC); (B, D, F, and H) anti-RNP IgG polyclonal antibody purified by traditional methods (TM- ion exchange chromatography followed by ammonium sulfate precipitation method). (G, and H) negative control. Original magnification 400×. The presence of bright green fluorescent inclusions indicates a rabies virus infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

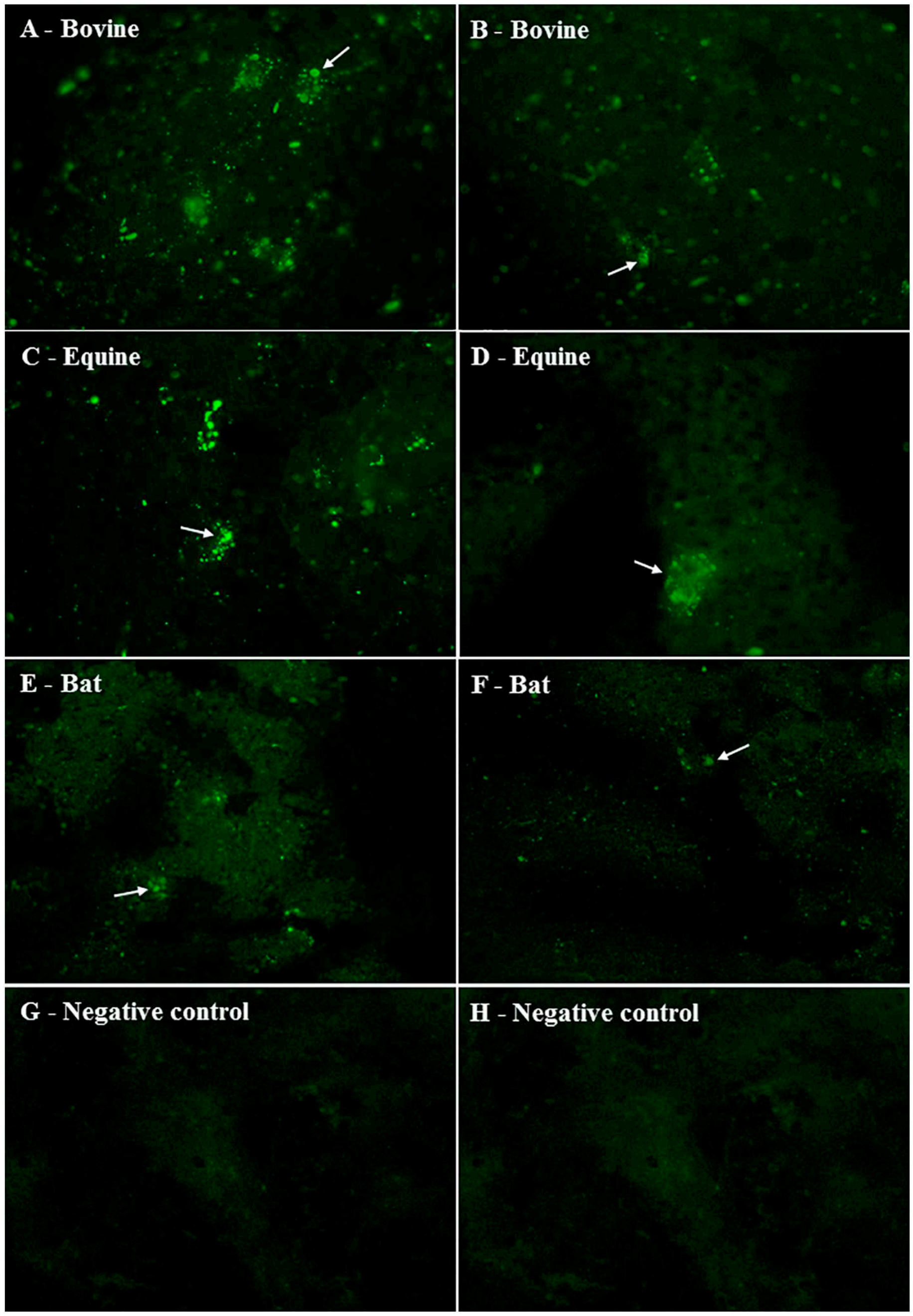

Fig. 5. Representative images of a touch impression of rabies RABV-positive animal brains testing by direct fluorescent-antibody assay. (A, C, E, and G) anti-RNP IgG polyclonal antibody purified by immunoaffinity chromatography on immobilized RNP (IAC); (B, D, F, and H) commercial reagent (Merck Life Science). (G, and H) negative control. Original magnification 400×. The presence of bright green fluorescent inclusions indicates a rabies virus infection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Antibody purification can be achieved by a range of methodologies, including precipitation, electrophoretic separations, filtration, and liquid and affinity chromatography (Grodzki and Berenstein, 2010; Roque et al., 2007). However, the choice of purification method depends on the intended application of the antibodies.

Traditionally, the ion exchange chromatography techniques associated with ammonium sulfate precipitation have been used to obtain RABV-specific FITC-labeled polyclonal conjugates for use in DFA tests (Caporale et al., 2009; Rupprecht and Nagarajan, 2015). This method is well established in our laboratory for the purification of IgG antibody. However, the purification methodologies based on ion exchange or adsorption fail to resolve complex protein mixtures to yield a homogeneous protein product from serum in a highly purified form. Additionally, this method allows for the partial purification of the protein of interest, which may compromise the efficiency of the immunodiagnostic assay. In this study, the purification of the horse anti-RNP IgG was performed by ion exchange chromatography followed by a homemade immunoaffinity chromatography on immobilized RNP in order to obtain specific antibodies with higher affinity.

Affinity chromatography-based methods are based on highly specific and reversible biological interactions between two molecules (e.g., between receptor and ligand or antibody and antigen). Affinity chromatography offers very high selectivity, involves minimal steps, and provides simplicity of approach and speed (Ayyar et al., 2012).

Antibody purity is essential because other contents in the source material may interfere with the detection process (Huse et al., 2002).

IgG antibodies from animal serum (rabbit, horse, and sheep) are easily purified by ion exchange chromatography under these conditions (Corthier et al., 1984). Thus, a QAE-column (anion exchange chromatography) was used for the isolation of horse IgG antibody. For this application, this method is satisfactory, and in regard to the 25 ml volume of serum taken from the horse, a noticeable amount of horse IgG can be obtained. Therefore, antigen-immunoaffinity chromatography was developed (the antigen RNP was covalently attached to solid media) to purify, with highly specificity, anti-RNP IgG antibodies. The conditions of the RNP-IAC column were evaluated, such as column capacity, recovery and reusability. Column capacity is an important factor in the evaluation of the efficacy of the antigen-immunoaffinity chromatograph (Ayyar et al., 2012). Thus, the column capacity of the RNP-IAC was 2.37 mg/ml. As a matter of comparison, the capacity of Hitrap Protein A was approximately 18.7 mg IgG/ml. We can note that the column capacity of the Hitrap protein A is significantly higher than that of RNP-IAC, because the commercial column can bind all IgGs strongly and not only IgG against RNP. The column volume of RNP-IAC used in this study was sufficient. However, for the RNP-IAC column, it is possible to purify more antibodies by increasing the column volume. The homemade RNP-IAC was highly stable compared with that in a previous report, in which the column capacity decreased to 75.0% after using it 6 times (Hu et al., 2013). Together, these results show that RNP-IAC retains its antibody-binding capacity.

Following the purification, antibodies with a satisfactory degree of purity were obtained. The results of the SDS-PAGE showed that proteins with a molecular weight of approximately 55 kDa were the horse IgG heavy chains, and bands between the molecular weights of 24 kDa were the horse IgG light chains, and both are compatible with the molecular weight of horse IgG (Sugiura et al., 2000). In indirect ELISA against RNP, the reactivity of the polyclonal IgG purified was high.

Since antigen affinity chromatography involved adjustment of the sample to a low pH at some point during the purification process, which may have resulted in the degradation of the antibody or may have affected antigen recognition, which are critical factors in increasing the efficacy and application of antibody in diagnostics. Therefore, the purified IgG antibodies were tested for their binding strength to their specific Ag (RNP) using an "avidity ELISA", which employed the chaotropic agent NaSCN and the antibody avidity obtained using the proposed methodology were compared with traditional techniques. We observed that the anti-RNP IgG antibodies purified by ion exchange chromatography followed by RNP-IAC exhibited high avidity values compared to IgG antibody isolated by traditional methods. Since only protein molecules containing the epitopes recognized by the immobilized antibody will be retained on the column, RNP-IAC has a very high resolution. However, by increasing the volume of the initial antiserum (120 ml) applied for purification of anti-RNP antibody by the traditional method, an avidity value that was similar to the antibody purified by IAC was verified.

The use of antibodies in a DFA test requires the conjugation of these proteins with FITC, which makes the reaction detectable. Thus, anti-RNP IgG conjugated to FITC exhibited an F/P ratio of approximately 2.8. F/*P* values of 3–10 are probably optimal for any particular IgG (The and Feltkamp, 1970). In a DFA test, the optimum dilution of prepared FITC-conjugated anti-RNP IgG was 1:30. The same titer was obtained with anti-rabies IgG purified by the traditional method; however, this was in samples starting from a larger volume of antiserum (120 ml). It is worth mentioning that Caporale et al. (2009) demonstrated that anti-RNP IgG–FITC purified from 25 ml rabbit serum hyperimmunized by the traditional method obtained a titer 1:400 for a DFA test application. Together, these data reinforce the work published by Bergmann-Leitner et al., 2008, which demonstrated that the choice of the optimal purification method depended on the species from which the sera/plasma were derived, since nonspecific Ig purification methods rely on the chemical properties of the antibody

The other criterion that was analyzed for the evaluation of the conjugates' quality was the absence of fluorescence in negative controls. In addition, we observed 100% agreement when comparing purified anti-RNP antibody with the anti-RNP IgG antibody obtained by the traditional method and the commercial one when using DFA in the sensitivity and specificity assays using CNS samples from different animal species.

5. Conclusions

In this report, a homemade IAC was developed for the purification of anti-RNP IgG with higher affinity antigen compared to the traditional method. In addition, we showed that the anti-RNP IgG antibody-FITC preparation obtained was suitable to use as a rabies diagnostic reagent by the DFA test.

Competing interests

The authors declare that they have no financial or personal relationships that may have inappropriately influenced them in writing this article.

Acknowledgments

We thank the Instituto Pasteur, São Paulo, Brazil for its financial support (IP 08/2018). The present manuscript was written based on a dataset from a monograph work of Jaqueline Santos and Gabriela Hidaka. We thank José Roberto Marcelino, MS (director of the immunology department at the Instituto Butantan/São Paulo/Brazil) and Graciane Maria Medeiros Caporale, PhD (researcher at the Instituto Pasteur) for the availability of the hyperimmune serum applied in this study.

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