

Characterization of a latex agglutination assay for the detection of Rheumatoid Factor

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ABSTRACT

One of the serologic criteria to diagnose Rheumatoid Arthritis (RA) is the determination of serum Rheumatoid factor (RF), which is an autoantibody targeting IgG. It can be detected by agglutination assays using commercially-available polystyrene latex-based reagents, which are marketed at relatively high cost. In low-income settings, homemade latex-based reagents can be a cost-effective alternative. Multilatex® is a registered trademark of polystyrene latex. It is produced at the Center of Biomaterials of the University of Havana (Havana, Cuba) for the development of diagnostic reagents. This work was aimed to obtain a polystyrene latex-based homemade system for RF detection, using Multilatex® and purified IgG as RF target. IgG was purified from commercially available purified human blood gamma globulin fraction, through affinity chromatography with protein G and adsorbed onto 800 nm-diameter Multilatex® polystyrene spheres. The RF detection system was calibrated to a detection limit of 8 international units/mL of RF in patient serum, and evaluated in an agglutination test with 30 positive and 30 negative RF human sera. The developed system behaved similar when compared to a commercial reagent of 100 % specificity and 100 % sensitivity, indicating that it could be developed for diagnosis of RA.

Keywords: agglutination assays, antibody, biomaterials, disease diagnosis, purification, rheumatoid factor

RESEARCH

RESUMEN

Caracterización de un ensayo de aglutinación con látex para la detección del Factor Reumatoide. Entre los criterios serológicos para diagnosticar la Artritis Reumatoide (AR) se encuentra la presencia en suero del Factor Reumatoide (FR), un autoanticuerpo que se une a la IgG. Este se puede detectar mediante ensayos de aglutinación con reactivos basados en látex de poliestireno, que se encuentran disponibles en el mercado aunque a precios relativamente elevados. En este contexto, los reactivos no comerciales pueden ser una alternativa rentable en entornos de bajos ingresos. El Multilatex® es una marca registrada de látex de poliestireno producido en el Centro de Biomateriales de la Universidad de La Habana (La Habana, Cuba), para el desarrollo de reactivos para diagnóstico. En este trabajo se describe la obtención de un sistema no comercial basado en látex de poliestireno para la detección del FR, que emplea Multilatex® e IgG purificada como blanco del FR. La IgG fue purificada a partir de una preparación comercial de la fracción purificada de gamma-globulina de sangre humana, mediante cromatografía de afinidad con proteína G, y se adsorbió a esferas de poliestireno Multilatex® de 800 nm de diámetro. El sistema se calibró a un límite de detección de 8 unidades internacionales por mililitro de FR en suero de pacientes y se evaluó mediante prueba de aglutinación con 30 sueros humanos positivos y 30 negativos a FR. El sistema obtenido se desempeñó de manera similar al compararse con uno comercial de 100 % de especificidad y 100 % de sensibilidad, por lo que el sistema desarrollado podría ser útil en el diagnóstico de la AR.

Palabras clave: ensayos de aglutinación, anticuerpos, biomateriales, diagnóstico de enfermedades, purificación, factor reumatoide

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Introduction

The Latex agglutination assay (LAT) is a simple, fast, instrumentation-less and easy to interpret technique, useful for the detection of the Rheumatoid Factor (RF) [1], which is an autoantibody targeting IgG [2] and one of the serologic criteria to diagnose Rheumatoid Arthritis (RA) [3].

Latex reagents consist of antigens or antibodies coupled to polystyrene latex spherical particles [4], which

systems could be either commercially available or homemade. The second one can be produced at lower costs and, advantageously, can be easily validated against the target population. At the same time, both polystyrene latex and latex-based reagents are relatively simple to produce at the laboratory scale and the in-house development of latex reagents can be a cost-effective option.

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In the efforts to develop a latex-based system to accurately detect RF, commercially available purified human blood gamma-globulin fraction was previously adsorbed to homemade 480 nm-diameter polystyrene spheres [5]. Multilatex® is a brand of polystyrene latex produced at the Center of Biomaterials of the University of Havana (Havana, Cuba), by a surfactant-free emulsion polymerization method [6]. The developed detection system was evaluated by LAT with RF positive and RF negative human sera and, in comparison with a commercial reagent, un-specific agglutination was detected in 40 % of negative sera that was avoided after serum-complement inactivation [5]. The commercial reagent was manufactured with 800 nm-diameter polystyrene spheres and purified IgG as RF target. In order to develop an equivalent homemade system using Multilatex®, in this work, 800-nm-diameter polystyrene particles [7] and affinity-purified IgG as RF target were used. The performance of the test obtained was evaluated in LAT with a panel of 30 RF-positive and 30 RF-negative human sera, and results compared with a commercial latex-based reagent for RF detection.

Materials and methods

Polystyrene latex

Multilatex® polystyrene latex spheres of 800 nm (mean diameter) were synthesized by the emulsifier free emulsion polymerization technique [6] and characterized as described [7].

Human serum samples

Human serum samples were collected at the Rheumatology Center of the Diez de Octubre Hospital (RC), Havana, Cuba. A commercial kit (RapiLat®-FR, HELFA Diagnostics®, Center of Immunoassay, Havana, Cuba) with 100 % clinical specificity, 100 % clinical sensitivity and detection limit of 8 international units (IU) per milliliter of RF in patient serum was used for serum classification and titration at the RC.

Purification of human IgG

The human IgG source was a commercially available purified human blood gamma-globulin fraction (human Immunoglobulins, hIgs). It is produced and commercialized by the Center for Production of Blood Derivatives Adalberto Pesant (Havana, Cuba), for the treatment of human immunoglobulin deficiencies.

The hIgs solution was dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.46 mM KH₂PO₄, pH 7.4). Human IgG was purified through affinity chromatography with protein G (GE Healthcare, Uppsala, Sweden), according to the manufacturers' instructions.

Affinity chromatography was performed using the AKTA FPLC System and UNICORN software version 3.0 (GE Healthcare, Uppsala, Sweden), for data acquisition and calculation of areas under the peaks.

Characterization of purified IgG

Enzyme-linked immunosorbent assays (ELISA) to test IgG were performed in 96-well flat-bottom microtiter plates (Maxisorp, Nunc, Denmark) coated with 0.1 mL of protein solutions at 10 µg/mL in

glycine-buffered saline (GBS; 100 mM Glycine, 150 mM NaCl, pH 8.2), at 4 °C for 18 h. After blocking with skimmed milk, plates were directly incubated with an anti-human IgG-alkaline phosphatase (AP) conjugate (Sigma, USA). Reactivity was revealed with p-nitrophenyl phosphate (Sigma, USA) according to the manufacturer's instructions. Absorbance was read at 405 nm (A_{405nm}) in a microplate reader (Thermo Electron Corporation, USA). All samples were analyzed in duplicate; results were expressed as mean absorbance units at 405 nm values. ELISA to assess IgM were similar but either GBS, PBS or PBS at pH 6.45 were used as coating buffers. A pre-coating step was also tested with 5 µg/mL Poly-L-lysine in PBS at 37 °C for 30 min.

ELISA to test the reactivity of human sera with the purified IgG were performed similarly, by incubating diluted sera at 37 °C for 1 h. GBS was used as coating buffer and reactions were developed with an anti-human IgM-AP conjugate.

For the dot blot immunoassay, 50 µg of antigens were vacuum-adsorbed onto 0.2 µm nitrocellulose membranes (Bio-Rad, USA) using a Manifold II S&S (Germany). After membrane blocking and incubation with the corresponding anti-human either IgM- or IgG-AP conjugate, reactions were developed with the 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt.

Analysis by SDS-PAGE [8] were performed on 7.5 % or 12.5 % polyacrylamide gels under non-reducing or reducing conditions, respectively.

Protein concentration determination

Protein concentration was determined with the BCA protein assay kit (Pierce Biotechnology, Rockford, USA) according to manufacturers' instructions.

Preparation of latex reagents

Adsorption on latex spheres was studied in GBS as reported [7]. Protein ranged from 1 to 5-fold excess of the amount required to saturate the calculated total particle surface area (i.e., protein concentrations from 0.18 to 0.89 mg/mL). Equations described in the Bangs Laboratories protocols for adsorption to microspheres were used for calculations [9]. The minimal protein concentration allowing particle saturation was set to produce 10 mL of the latex reagent, which was further calibrated with the World Health Organization International Reference Preparation NIBSC 64/2 (Rheumatoid Arthritis Serum) [10] using Bovine Serum Albumin (BSA, Sigma, USA). Latex reagents were stored at 4-8 °C until use.

Latex agglutination test

A mix of 20 µL of sample and 20 µL of latex reagent was gently agitated for two min, followed by LAT results scoring as positive or negative if clumps were visible or not, respectively. Positive sera were serially diluted with 0.9 % NaCl (w/v) and sera titers were regarded as the maximal dilution giving positive agglutination. The RapiLat®-FR commercial kit was used as control. The assays included the negative and positive controls supplied with the commercial kit. All serum samples were analyzed in duplicate and in case of uncertainty, a third test was performed.

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Results and discussion

Human IgG was purified from a solution of hIgs through affinity chromatography, as shown in the affinity-chromatography profile (Figure 1). The calculated areas under the peaks in the chromatogram indicate that 97.2 % of total proteins in the hIgs solution were IgG, whereas other proteins were represented the 2.7 % of total protein content.

The SDS-PAGE analysis of the purified IgG fraction revealed the typical IgG pattern, consisting of bands of 50 and 25 kDa, corresponding to heavy and light immunoglobulin chains, respectively (data not shown).

The IgG fraction was corroborated in the purified protein preparation by ELISA signals at least three-fold higher than those registered with an unrelated control protein (Figure 2A) and at the level of those recorded for the hIgs solution. In this experiment, GBS was used as coating buffer to favor IgG binding at isoelectric point to polystyrene plates.

In a previous work [5], there was developed a RF detection reagent consisting on hIgs adsorbed to polystyrene spheres. In that case, 40 % of unspecific agglutination was observed, which was avoided after serum-complement inactivation. These false-positive results were then attributed to serum-complement activation, due to the presence of IgM in the hIgs solution used as RF target. For this reason, in the present work, a series of experiments were conducted aimed to detect the possible presence of IgM in the purified IgG solution. As expected, the IgG purification method applied rendered negative results for IgM presence in all the experiments. This confirmed that purified IgG solution was IgM-free or at least that IgM levels were below the detection limits of the assays. These experiments included ELISA tests using GBS (pH 8.2), PBS (pH 7.4) or PBS at pH 6.45 in the coating step. The pH was lowered to favor IgM binding to polystyrene plates at isoelectric point. The use of a pre-coating step with Poly-L-Lysine was included to favor binding of IgM, of higher carbohydrate content than IgG. A dot blot immunoassay testing 50 µg of antigen also rendered negative results for IgM presence, whilst confirming IgG content (data not shown). Antigenicity of purified IgG was demonstrated by the dilution-dependent recognition of the antigen by three RF positive sera. ELISA signals at blank (GBS) level were recorded for three RF negative sera (Figure 2B).

Saturation of polystyrene sphere surfaces was found at 26 µg of purified IgG per mg of spheres, in close agreement with theoretical calculations [9]. The developed latex reagent was calibrated to a detection limit of 8 IU/mL, to match the detection limit of the commercial RF detection kit used for comparison in the present study. After calibration, negative and positive reactions were verified with control reagents included in the commercial kit.

Negative LAT results were recorded for the 30 RF negative sera using both the commercial kit and the detection system prepared in the present work using Multilatex® and purified IgG as RF target. The use of purified IgG circumvented the unspecific agglutination with RF negative sera previously obtained with the 480 nm-diameter polystyrene spheres-based

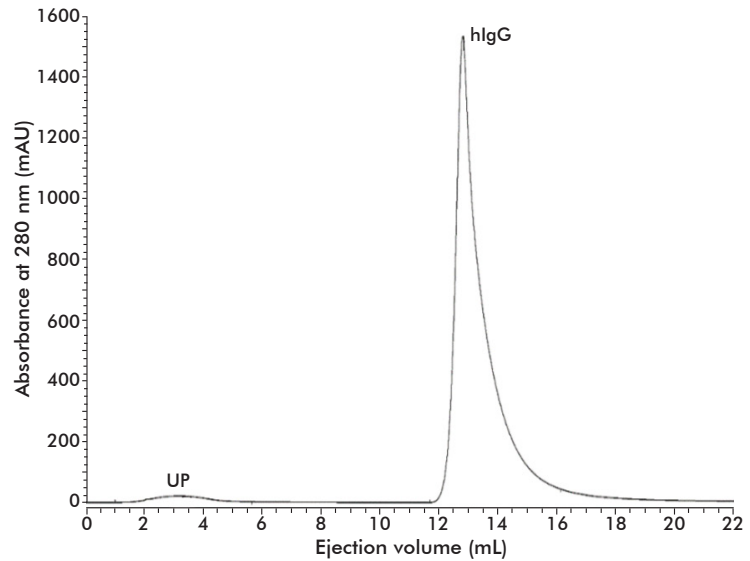


Figure 1. Protein G affinity-chromatography profile of the human immunoglobulins (hlg) solution. The graph shows the absorbance at 280 nm ($A_{280\text{nm}}$; mAU) vs the elution volume in mL. The hlgG peak contained 97.2 % of total proteins whereas unbound proteins (UP) represented just 2.7 % of total protein content. Protein percentages were calculated from the areas under the peaks.

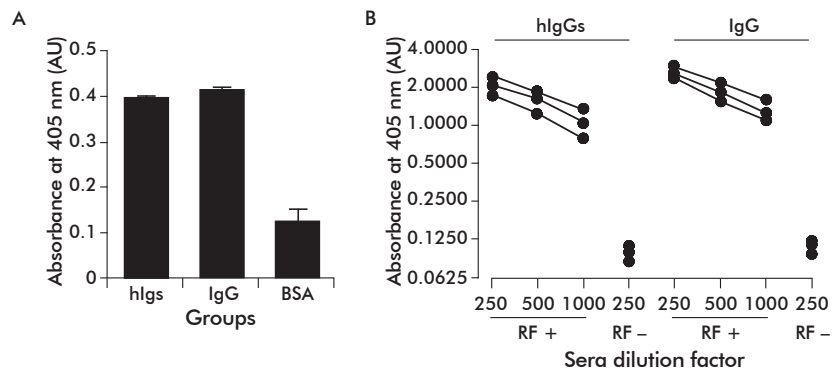


Figure 2. Characterization of purified IgG fraction (IgG) by ELISA. A) Reactivity with anti-human IgG antibodies. Protein solutions of human immunoglobulins (hIgs) and bovine serum albumin (BSA) were used as positive and negative controls, respectively. Protein solutions were evaluated by duplicate; mean values are represented. B) Reactivity with three RF positive (RF+) and three RF negative (RF-) human sera. The hIgs solution was used as positive control. Antigens were recognized by RF positive sera (results for each serum sample are connected by lines) in a dilution-dependent manner, whereas $A_{405\text{nm}}$ values recorded for RF negative sera were at the blank level. All sera were evaluated in duplicate; mean values are represented.

reagent [7]. This emphasized the importance of IgG purity on the performance of polystyrene latex-based reagents to detect RF.

As shown in Figure 3, all FR positive sera gave positive results with both latex reagents. Serum titers measured with the commercial reagent were similar ($p > 0.05$) to those informed by the RC. Titers measured with the homemade detection system were similar ($p > 0.05$) to those measured both at the RC and confirmed in the present work with the commercial system tested.

In summary, a latex-based system was implemented to detect RF using Multilatex® as solid support and purified hIgG, under the defined and tested experimental conditions. It gave results similar to those

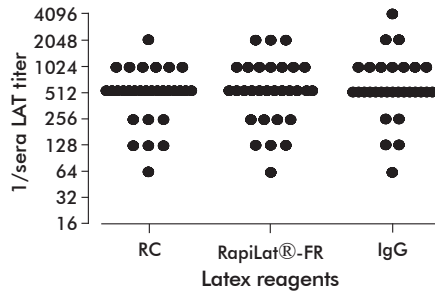


Figure 3. Latex agglutination test (LAT) titers of Rheumatoid Factor (RF) in 30 positive human sera. Titers were measured with the homemade detection system developed in the present work using purified IgG and Multilatex® (IgG). The commercial kit RapiLat®-FR (HELFA Diagnostics®, Havana, Cuba) was used for comparison, guaranteeing 100 % sensitivity and 100 % specificity. Results reported by the Rheumatology Center at the Diez de Octubre Hospital (RC), Havana, Cuba were also included in the comparison. Data were analyzed with the non-parametric Kruskal-Wallis test and no overall statistically significant differences were detected among data sets ($p > 0.05$). Mean values are represented by the horizontal bar.

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obtained with a commercial system for RF detection, when tested against a panel of 30 positive and 30 negative human sera. Future work will address the validation of the system and whether could it be possible to implement this system for RA diagnosis in the clinics.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.