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A fast and straightforward method for the purification of Anti- Immunoglobulin G (IgG) for Coombs Wright assay

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ABSTRACT

Introduction: Immunoglobulin G is the most abundant immunoglobulin in human blood, comprising approximately 75% of serum antibodies. Human IgG is a glycoprotein and can be an antigen for other mammals. Antibody-sensitized red blood cells (RBCs) and complement components can react with anti-human globulins resulting in their agglutination. This study aimed to prepare an antiserum against human IgG for deploying in Coombs Wright and cross-match for rare blood groups. **Methods:** After isolation of serum from healthy blood donors, serum proteins were precipitated using ammonium sulfate. Consequently, tangential flow filtration and ion-exchange chromatography were applied to purify IgG. SDS-PAGE and Bradford protein content assay was conducted to evaluate the quality and the concentration of the purified IgG. Rabbits were weekly injected with different amounts of the protein four times. Then, sera were obtained from the immunized mice, and total IgG was purified. Finally, the Coombs Wright test was performed on samples from brucellosis patients to validate purified IgG antibody quality. **Results:** Electrophoresis and Bradford assay results showed that the purified protein had considerable high purity and quantity. Protein bands of reducing and the non-reducing SDS-PAGE showed high purity of the protein along with a protein yield of 2.2 mg/L. Coombs Wright tests using the rabbit anti-human serum had a comparable result with available commercial anti-human immunoglobulin. **Conclusion:** The results indicated that our method for the purification of IgG was suitable for anti-human globulin preparation. This antibody can also be used in clinical diagnostic tests such as Coombs Wright, cross-match, and blood types evaluation with weak Rh or Du antigens.

INTRODUCTION

Human brucellosis is a bacterial disease transmitted via contact with infected animals or contaminated dairy products [1]. Four *Brucella* species, *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis*, cause brucellosis. Two species *B. Abortus* and *B. canis*, cause a mild infection accompanied by non-caseating granuloma in the liver and reticuloendothelial system [2], while infection with *B. suis* results in a more severe infection accompanied by caseating granuloma and purulent lesions; *B. melitensis* causes the most severe form of the disease [3-8]. Brucellosis is a multisystem infection affecting various organs such as the reproductive system, liver, heart, and central nervous system. Chronic brucellosis can cause a local or systemic disease.

The best detection method is the blood culture, cerebrospinal fluid, synovial fluid, and bone marrow specimens. However, isolating the bacteria by culture is not always possible, especially during the chronic disease phase [3,4,9]. There are several serological tests for the detection of brucellosis, including **(i)** standard tube agglutination (STA) or Wright's test, which detect IgM and IgG, **(ii)** 2ME agglutination test that detects IgG, and **(iii)** Coombs Wright and complement fixation assays that detect IgG. Other tests such as radioimmunoassay, ELISA, Rose-bengal test, ring test,

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and slide agglutination tests are also available [10-13]. However, the Wright test sometimes becomes negative despite a positive blood culture. In such cases, the blocking IgG antibodies occur without the potential of agglutination. Anti-human globulins are used to detect these antibodies. Coombs Wright test detects the acute and chronic brucellosis [14]. For preparing anti-human globulin, purified human IgG should be used as an antigen and injected into a suitable host such as rabbits [15]. After blood collection and purification of anti-human globulin-containing serum, the anti-human globulin can be used in coombs Wright and cross-match tests [16].

Antibodies are gamma globulins found in the blood and other body fluids of vertebrates and identify or neutralize foreign antigens such as bacteria or viruses [15]. In mammals, IgG is the most crucial serum immunoglobulin. Therefore, the serum is the primary source of IgG isolation [16]. Human IgG is a 150-KD glycoprotein and is an antigen for animals like rabbits. Antigens with a molecular weight of more than 100 KD are usually potent antigens and can stimulate specific immune system responses [16]. Anti-human IgG, an antibody against human IgG, is produced by various laboratories. This antibody has a wide range of applications in diagnostic assays such as Coombs Wright, direct and indirect coombs, ELISA, and immunofluorescence assays. In the present study, we focused on developing a robust, low cost and time-saving method to produce an efficient anti-human globulin.

MATERIALS AND METHODS

Clinical samples. Five blood samples were obtained from healthy individuals at Day General Hospital, Tehran, Iran, following the participants' informed consent. The blood samples were then untagged and re-labeled by numbers as a sample identifier.

IgG purification from human serum. We collected blood samples from healthy individuals with no current infection and immune system diseases. One hundred-fifty (155) ml of blood was centrifuged at 850 g for 30 min at 4° C, and 80 ml serum was separated. An equal volume of 0.15 M NaCl (Merck, Cat. No. 106404) was added to the sera, and the mixtures were placed on a stirrer at 4° C. One hundred sixty (160) ml of saturated ammonium sulfate was used to precipitate the proteins, followed by centrifugation of the mixtures at 800 g at 4° C for 15 to 30 min. The supernatant was discarded, and a 50% saturated ammonium sulfate solution in the ratio of 1:2 was added to the precipitate. NaCl 0.15 M (one-third of the initial volume of serum) was added to the precipitate [12], and tangential flow filtration (Labscale TFF System, Merck) dialysis was performed using a 100 KD filter (Merck, Pellicon® XL50 with Biomax® 100 kDa Membrane, C screen, 50 cm², Cat. No. PXB100C50) versus PBS (Phosphate-buffered saline, Gibco, Cat. No.18912014).

Ion-exchange chromatography (DEAE Sepharose® Fast Flow, GE17-0709-01 Sigma) was used for further purification of IgG. After equilibration of the chromatography column using 0.07 M PBS, the protein mixture was applied to the column, and several resulting fractions were collected. In order to concentrate the samples, TFF was used using a 100 KD filter and PBS (Na2HPO4; Merck, Cat. No 106586, NaH2PO4; Merck, Cat. No 106346. pH=7.6). Nessler test was used to evaluate the removal of ammonium sulfate from the purified protein. Briefly, 20 µL of Nessler reagent and 25 µL KOH was added to 150 µL of the samples. Whenever the samples contain ammonia, a yellow-colored compound appears with the intensity proportional to the ammonia concentration.

SDS-PAGE was used to evaluate the purified antibodies quality, both after the initial precipitation and after the final step to reach high-quality purified human-IgG. The SDS-PAGE gel with a reducing agent (2-mercaptoethanol, 1%) was prepared. Bradford assay was used to determine the concentration of the purified antibody.

Rabbit immunization with purified IgG. Two hundred-fifty µg of the purified protein was dissolved in 1 mg aluminum hydroxide gel, and the mixture was placed on a shaker at 100 RPM for 30 minutes. Table 1 shows the antigen composition for injection.

Table 1. The components of the antigen for injection.

Component	Volume (µL)
Normal saline (0.85%)	849
Aluminum hydroxide	37
Human globulin (antigen)	114
Final volume	1000

New Zealand white rabbits (male, 2-3 kg) were injected weekly, with 1 mL of the antigen for four weeks. The first injection was subcutaneous, and the others were intramuscular into the hind limb [12]. One week after the last injection, blood samples were collected. The samples were centrifuged at 800 g for 30 min at 4°C to separate the serum-containing anti-human IgG.

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Purification of anti-human IgG. Purification of anti-human IgG was performed according to the protocol mentioned above in the section "IgG purification from human serum". The absorbance of samples from each purification step was determined using Nanodrop (Thermo Scientific, Waltham, MA, USA). The anti-human IgG purity was determined using SDS-PAGE with a reducing agent (2-mercaptoethanol, 1%).

Coombs Wright test. Ten brucellosis-positive and five negative sera were obtained from Day Hospital, Tehran, Iran. The sera were serially diluted 1:20 to 1:1280 with normal saline 0.85%. Five hundred µL of Wright tube test antigen (produced at Pasteur Institute of Iran) was added to each tube. The tubes were incubated at 37°C for 24 h, and the results were interpreted. All tubes with agglutination were excluded, and non-agglutinated tubes were centrifuged at 1000 g for 10 min at RT. The supernatants were discarded, and the precipitate was resuspended in 1 mL PBS and centrifuged three times at 1000 g for 10 min at RT. The final precipitate was resuspended in 0.9 mL PBS, and 0.1 mL anti-human globulin was added to it. The mixture was incubated at 37 °C for 24 h, and the results were interpreted.

RESULTS

Human IgG purification. After ion-exchange chromatography and TFF, the purified antibody was applied to reducing SDS-PAGE, and two bands of 25 and 50 KD were observed (Fig. 1), indicating the high purity of the isolated antibody. The purified protein concentration was determined after the removal of ammonium sulfate using a 100 KD filter. Protein concentration was 19.6 and 17.8 mg/mL using Nanodrop and Bradford assay, respectively.

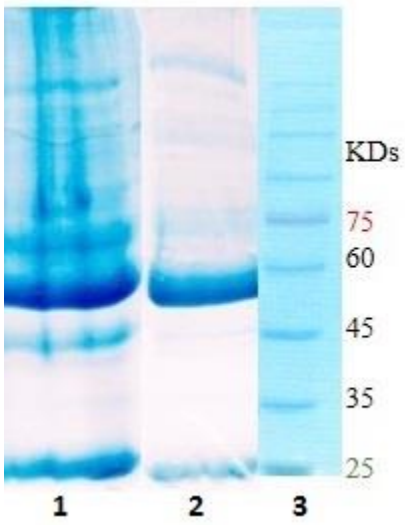


Fig 1. Evaluation of the purified antibodies. Protein electrophoresis after precipitation by ammonium sulfate and diafiltration by Tangential flow filtration. Lane 1: protein sample after precipitation using ammonium sulfate, Lane 2: protein sample after diafiltration with 30 kDa cut-off filters. Lane 3: Protein ladder (25-165kDa).

Rabbit anti-human IgG purification. Ten fractions were collected during ion-exchange chromatography, and the protein concentration of each fraction was determined. The sample fractions were investigated using a UV detector (Amersham Biosciences AKTA UV-900 Monitor). The device worked based on optical density (OD) measurement at 280 nm. Once the protein concentration increased and a peak appeared on the monitor, the fraction was collected (Table 2). The purity of the fractions was evaluated using reducing polyacrylamide gel electrophoresis. Figure 2 shows that the proteins are highly pure.

Coombs Wright. Nine serial dilutions from the patients' sera were prepared, and the last dilution with agglutination was considered the highest titer. After performing the Coombs Wright test, 100 µL of the purified anti-human globulin was added to the non-agglutinated tubes. BIO-RAD (Anti-IgG, polyclonal, rabbit) anti-human globulin was used to compare the produced anti-human globulin (Table 3).

DISCUSSION

Identification of brucellosis is still a challenge despite the development of various methods. Blood and body fluid cultures are not sensitive enough, need special culture media, and are time-consuming. Moreover, the bacterial smear is not specific since brucellosis bacteria are morphologically similar to *Chlamydia abortus* [17].

Many attempts to develop a suitable diagnostic method for brucellosis have reported different results. Therefore,

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using a cost-benefit, rapid, and effective method for detecting brucellosis is a priority.

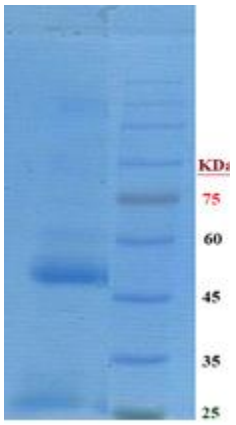


Fig 2. Evaluation of the purified anti-human globulin after ion-exchange chromatography and tangential flow filtration with 100 kDa cut-off filters using reducing polyacrylamide gel electrophoresis.

Table 2. The protein concentration of each fraction after chromatography measured by Bradford assay and absorbance at 280nm

Method	Protein concentration (mg/ml)	
Ion-exchange chromatography	Bradford assay	Absorbance at 280 nm
Fraction 1	1.11	1.2
Fraction2	4.16	4.7
Fraction 3	16.23	17.5
Fraction 4	20.01	22.2
Fraction 5	19.91	21.7
Fraction 6	18.89	20.98
Fraction 7	18.21	20.7
Fraction 8	12.82	13.8
Fraction 9	1.79	1.9
Fraction 10	0.61	0.062

Table 3. Comparison of Coombs Wright test results with prepared anti-human globulin and commercial anti-human globulin

Sample	Anti-human globulin produced in this study	Commercial anti-human globulin
Positive control	1/160 (4+)	1/160 (3+)
Negative control	(-)	(-)
Sample 1	1/320 (4+)	1/320 (3+)
Sample 2	1/160 (4+)	1/160 (3+)
Sample 3	1/320 (4+)	1/320 (3+)
Sample 4	1/80 (3+)	1/80 (4+)
Sample 5	1/320 (2+)	1/320 (2+)
Sample 6	1/160 (3+)	1/160 (3+)
Sample 7	1/160 (3+)	1/160 (3+)
Sample 8	1/80 (3+)	1/80 (3+)
Sample 9	1/640 (4+)	1/640 (4+)
Sample 10	1/40 (4+)	1/40 (4+)
Sample 11	(-)	(-)
Sample 12	(-)	(-)
Sample 13	(-)	(-)

Serological methods can also diagnose brucellosis but have inherent limitations. These methods via detecting antibodies might confirm the infection; however, the antibody concentration is sometimes below the detection limit [18]. Zargar et al. (2009) examined sera from 45 brucellosis patients using the Wright test at Tehran, Iran, among which 32 patients (71.11%) had antibody titers ≥1/160, while 28.89% were negative [19].

The tube agglutination test is less sensitive for detecting IgG (especially IgG3 and 4) than IgM. 2ME test (which eliminates IgM) in conjunction with Coombs

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Wright will provide more accurate results on the patient's condition [20]. Chegini et al. (2014) evaluated antibodies in 312 patients using Wright, Coombs Wright, and 2ME and reported 29.5%, 29.9%, and 21.1% of seropositivity, respectively. Their results indicated that Coombs Wright was a reliable screening test with a lower cost [21]. We also used Coombs Wright with our produced anti-human globulin to diagnose brucellosis in several patients. Our results indicated that the new anti-human globulin performed better than the commercial one. In fact, the dilutions were 4+ and 3+ with our anti-human globulin and the commercial one, respectively. The result of the tests are scored from 0 to 4+, i.e., 0 (no agglutination), 1+ (25% agglutination), 2+ (50% agglutination), 3+ (75% agglutination) or 4+ (100% agglutination).

Gómez et al. (2008) evaluated several serological tests in 2008. They used Rose-bengal, microagglutination, coombs, and immunocapture-agglutination methods, all of which were positive for all the patients. They also used the ELISA to evaluate the antibodies. IgG, IgM, and IgA were negative for 3, 10, and 1 patient, respectively. They concluded that ELISA is not more sensitive than the other serological methods for diagnosing brucellosis [22].

Kerr in 1994 and Walker in 1996 used gel filtration chromatography for the purification of immunoglobulins. This method is not suitable for IgG purification, and other complementary methods are required [23, 24]. We used TFF and ion-exchange chromatography and prepared a high quality and pure anti-human globulin. Besides, tangential flow filtration required less time than dialysis, and using 30 and 100 KD filters resulted in a more pure product. Electrophoresis and Bradford assay results also showed that the quality and quantity of the purified protein was remarkable. Protein bands on reducing and non-reducing SDS-PAGE also indicated that the whole immunoglobulins were of high purity. Finally, the Coombs Wright test using our produced anti-human globulin showed better results than the test using commercial anti-human globulin.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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