ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2015; 14(1):105-112.

Development of a New Immunochromatographic Assay Using Gold Nanoparticles for Screening of IgA Deficiency

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Received: 24 November 2013; Received in revised form: 3 February 2014; Accepted: 2 March 2014

ABSTRACT

A new competitive immunochromatography (ICG) strip test based on polyclonal antibody (pAb) conjugated with gold nanoparticles (NPs) was developed and its applications for primary screening of immunoglobulin (Ig) A in serum were evaluated. Nanocolloidal gold as the detection reagent, with an average particle diameter of 20 nm, was synthesized and labelled pAb.

The antibody-nanocolloidal gold probe was applied on the conjugate pad, and human IgA was immobilized on a nitrocellulose membrane as the capture reagent to prepare the ICG strip test. It took only 10 minutes to accomplish a semi-quantitative detection of serum IgA in this assay.

In the optimized investigational conditions, the ICG strip test could distinguish human serum IgA in the range from 1 to 270 ng/mL with a detection limit of 5 ng/mL.

The reliability of testing procedures was examined by performing the ICG strip test with 11 serum samples and comparing the results with those obtained via enzyme-linked immunosorbent assay (ELISA). The ICG strip was sufficiently sensitive and accurate for a rapid screening of IgA in human serum.

Keywords: IgA deficiency; Immunoglobulin A; Immunochromatography; Gold nanoparticle; Screening test

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INTRODUCTION

Immunoglobulin (Ig) A is the second dominant immunoglobulin isotype in blood circulation following

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

IgG that can be found in both monomeric and polymeric forms. Monomer IgA1 subclass is mainly found in the blood circulation, whereas dimeric IgA2 subclass is the foremost immunoglobulin in mucosal secretions. Although IgA functions in human blood have been described in 1953 but still many abilities are not clearly understood.[1,2]

IgA deficiency can be defined in patients older than four years of age as decreased or absent level of serum IgA in the presence of normal serum levels of IgG and IgM, in whom other causes of hypogammaglobulinemia have not been proven.[3] Total IgA deficiency (IgAD) is defined as an undetectable level of serum IgA at a value under five mg/dL (0.05 g/L or 5×10^4 ng /mL) in humans while partial IgAD refers to detectable but reduced levels of serum IgA which are more than two standard deviations below normal age-adjusted means.[4-6] The frequency of this condition in general population varies between 1: 400 and 1: 3000 in different countries.[7] Smaller proportion of these patients will be diagnosed during childhood, however; the proportion will increase over the years. This condition may be associated with an IgG subclass deficiency with increased probability of respiratory and digestive tract infections, allergies and autoimmune diseases.[8-13] During childhood, IgA deficient patients, may also be referred to the clinic due to symptoms of allergy, autoimmune diseases and tumors.[1] Anaphylactic reaction to transfusion of IgA-containing blood products due to the presence of anti-IgA antibody in some of the people's plasma is of more dangerous complications.[14,15] Thus, it seems that serum IgA measurement for every blood donor and recipient should be done for diagnosis and registration; and IgA deficient patients should be transfused only with IgA-free blood or components, which have been obtained from similar blood donors.[16]

The ICG assay, immunostrip assay or lateral flow assay was established at the end of the 1960s for the first time. It was originally developed for evaluating the existence of serum proteins. The first home-made ICG assay was established in1976 to detect the levels of human chorionic gonadotrophin (hCG) in the urine.[17] This assay is more based upon the antigen-antibody interaction for the rapid detection of target analyte.[18-25] Colloidal gold nanoparticles (NPs) have been used as a sensitive detector probe to establish one- step strip tests. Gold NPs with the diameter smaller than 15 nm were found to be too small for generating a strong color, hence nanocolloidal gold with a diameter of 20 nm has been used for developing ICG strip tests. Furthermore, colloidal gold NPs larger than 60–70 nm after storage at 4 °C for several days could be easily self-aggregated.[26] The intraparticle balance existing between electrostatic repulsion and London–van der Waals attraction contributes to the formation of the colloidal gold NPs. During the conjugation, immunoglobulins are directly attached on the surface of NPs by non-covalent reactions, such as London–van der Waals force and hydrophobic interactions.[26] There is a close association between the size, power of the color and quality of the colloidal gold NPs.[25,27]

Nowadays, biosensors are considered as supreme instruments, available in the health care system, particularly for on-site diagnosis and monitoring of diseases.[28-30] Immunchromatogrphy (ICG) assay is one of the promising tools in the development of easy-handling biosensors that has practical advantages both in clinic and basic sciences. The ICG method is used widely mainly because of its short procedure time, simple implementation, requiring no special instruments and no trained technicians, and low cost.[17] The aim of this study was to develop a polyclonal antibody (pAb)-nanogold-based ICG assay for screening patients with IgA-deficiency.

MATERIALS AND METHODS

Reagents

Anti human IgA goat polyclonal antibody (I0884 α-Chain specific), purified human IgA (I1010), tetrachloroauric (HAuCl 4) and sodium citrate, were obtained from Sigma Chemical Company (St. Louis, MO, USA). ELISA plates (96 wells) and other plastic ware were obtained from Nunc (Maxi-sorb, Roskilde, Denmark). Rabbit anti-goat IgG (H+L), was purchased from Thermo Scientific Company (31105-Rockford, USA). High-flow nitrocellulose membranes (HF135), glass fibers, and absorption pads were purchased from Millipore Corporation (Billerica, MA, USA). All the other chemicals used in the present study were either analytical pure or with highest quality.

Preparation of pAb–gold NPs Conjugate

Gold NPs with an average diameter of 20 nm and

antibody-gold NP conjugates were prepared according to the method described in our previous work[20] that is mentioned here briefly. The chloroauric acid solution (100 mL, 0.01% w/v HAuCl4) was heated to boiling, and then %1 sodium citrate solution was added with constant stirring. The solution was cooled down when the solution's color changed from light yellow to purple-red. The pH was adjusted to 8.5 by 0.01 M potassium carbonate, and 0.01% (w/v) sodium azide was added. The obtained NPs solution was stored at 4°C in a dark-colored glass bottle for several months. The diameter of colloidal gold NPs was checked with a transmission electron microscope (JEOLl, JEM-2100). The colloidal gold solution was scanned by spectrophotometer between 400 and 600 nm.

To prepare pAb-gold NP conjugates, 120 µg of anti-human IgA (anti IgA Ab) was added to 4 mL pH-adjusted gold NP solution and mixed immediately. The mixture was stirred gently overnight, and then 10 mL of 10% BSA solution was added to block the residual surface of the gold NPs, stirred 20 min at room temperature and centrifuged at 15,000 ×g for 45 min at 4°C for three times. After the last centrifugation, the pellet was re-suspended in 2 mL phosphate-buffered saline (pH 7.2, 0.01 M containing 1% BSA and 0.05% sodium azide), and the optical density was adjusted to 8.0 at 520 nm with dilution buffer. Then anti-IgA coated colloidal gold NPs was stored at 4°C until use.

Preparation of the ICG Test Strips

A schematic diagram of the ICG test strip is shown in Figure 1 a. An ICG test strip consists of a sample pad, a detector conjugated pad, an absorption pad, and a nitrocellulose membrane with test and control lines. The sample pad was immersed in buffer (pH 7.4) containing 20 mM sodium phosphate, 1% (w/v) BSA, 0.5% (v/v) tween-20, and 0.05% (w/v) sodium azide and dried for 1 h at 50 °C. The absorption pad was used without treatment. The conjugate pad was treated with a 5% (w/v) sucrose solution in water and dried at 37 °C. Then, 4 µL/strip pAb-gold NPs as label was applied to the conjugate pad and dried for 30 min at 37 °C. Subsequently, 1 µL rabbit anti-goat secondary antibody (1 mg/mL) and IgA antigen (2 mg/mL) diluted in PBS (0.01 M, pH 7.2) were coated onto a nitrocellulose membrane as two discrete zones, one for control and the other for test with Camag Linomat 5 automatic TLC sampler (Camag, 4132 Muttenz, Switzerland) and dried at room temperature. The reason of drying the membrane at room temperature was to fix the antibody and the antigen to the nitrocellulose. Then the four parts of the ICG test strip were assembled on a polystyrene membrane with an adhesive surface and the strips were cut with 70mm in length and 5mm width. The strips were sealed in a plastic bag containing desiccant gel and stored at 4°C until use. The IgAD-ICG test strips with the immobilized purified human IgA and rabbit anti-goat secondary antibody preserved their stabilities within six months after storage at 4 °C.[20]

Sample Collection and Analysis

Blood samples were obtained voluntarily from 11 patients who had referred to the Immunology, Asthma, and Allergy Research Institute of Tehran University of Medical Sciences according to the Declaration of Helsinki and the protocol that was approved by the Institute. Every human blood sample was transferred into a test tube. After incubation at room temperature for 1 h, the test tube was centrifuged for 10 min at 1,200 ×g. Then the serum was transferred to a new vial and stored at -20 °C until use.

The precise concentration of IgA in the 11 different serum samples was measured by IgA ELISA kit (K1316 Sanquin, Netherlands) and their results were compared to the IgAD-ICG test strips with 100µl of the samples (Table 1).

Table 1. Serum IgA determination by IgAD-ICG test strip and ELISA

Case No.	ELISA Serum IgA (mg/dL)	IgAD-ICG test strip Visual result
1	64	Neg.
2	40	Neg
3	23	Neg
4	0.4	Pos.
5	2.2	Pos.
6	1.4	Pos.
7	137	Neg
8	108	Neg
9	340	Neg
10	1.6	Pos.
11	220	Neg

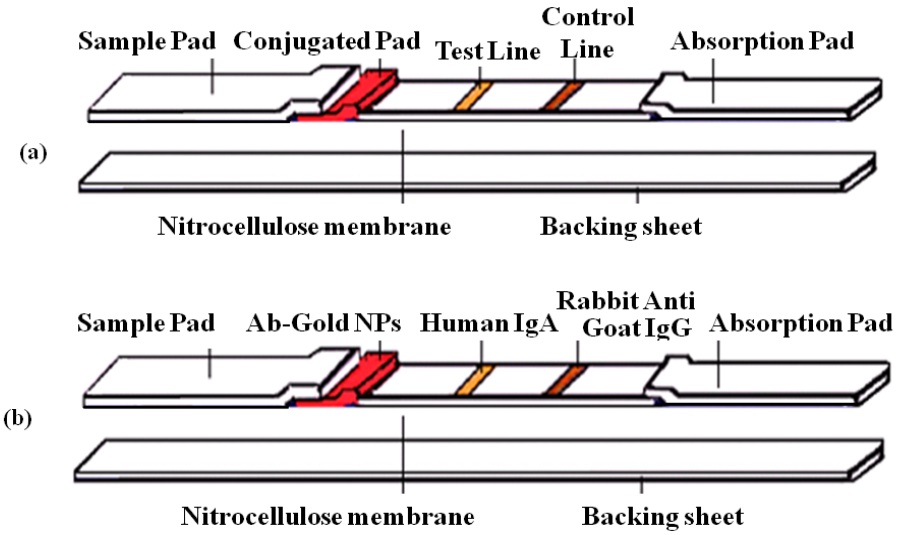

Figure 1. (a) A typical ICG test strip. The strip consists of a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorption pad. (b) IgAD-ICG test strip.

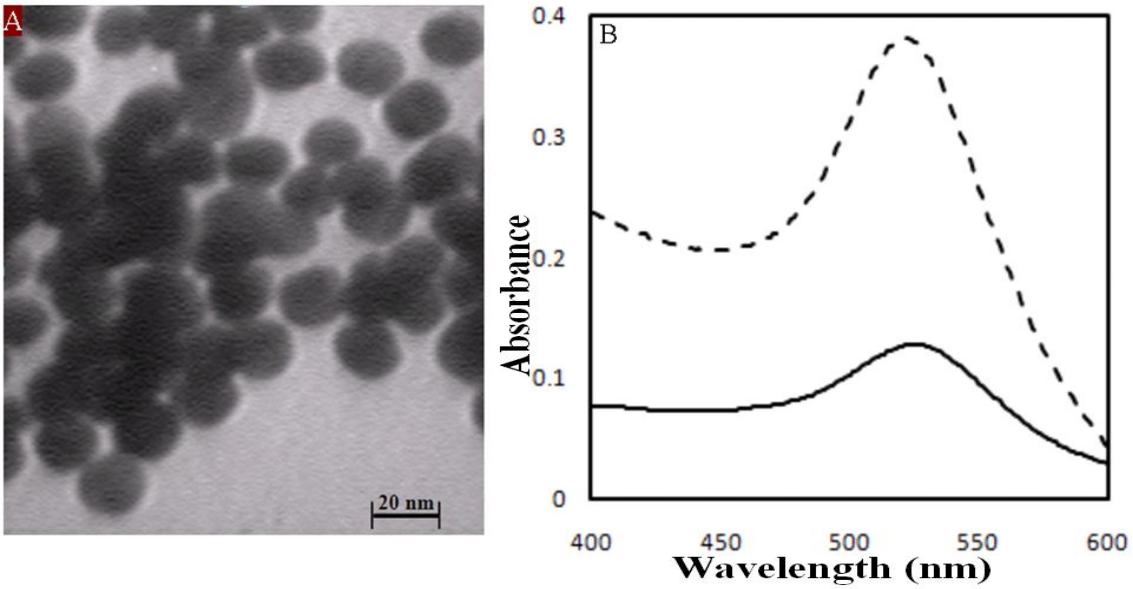

Figure 2. (A) Transmission electron microscopy of colloidal gold particles. The average diameter of the particles was approximately 20 nm. (B) The UV–vis spectra of the colloidal gold solution before and after conjugation to antibody (Dashed line; gold NPs, solid line; Ab-gold NPs conjugate).

RESULTS

Characterization of the pAb-gold NP Conjugate

In order to synthesize nanogold particles, the tetrachloroauric acid (HAuCl4) was reduced in the presence of trisodium citrate as reducing agent. The formation of colloidal gold was screened using UV–vis spectroscopy method (200–700 nm) by means of a double-beam spectrophotometer (Shimadzu, model 1.70, GBC) running at one nm. Antibody–colloidal gold conjugates were also monitored immediately after preparation.

Figure 2 A shows the diameter of the colloidal gold NPs obtained by the transmission electron microscope (TEM). The UV-vis spectroscopic analysis showed a maximum absorbance at ~519 nm which is caused by the surface plasmon resonance of colloidal gold particles (Figure 2 B). The surface plasmon band

was expanded and led to red shift due to the interaction between the antibody and gold colloidal particles. TEM images revealed that the average diameter of colloidal gold particles was approximately 20 nm. The results from both experiments offer the close distribution of colloidal gold which provides a basis for probe preparation and strong signal production in the test strip.

Construction of the IgAD- ICG Test Strip

Gold-labeled antibody probe is applied to construct an ICG test strip for IgA. Figure 1b represents a schematic diagram of the preparation of the IgAD-ICG test strip.

One microliter of different concentrations of purified human IgA antigen (0.5, 1, and 2 mg/mL) and 1µg/µL of rabbit anti goat secondary antibody were

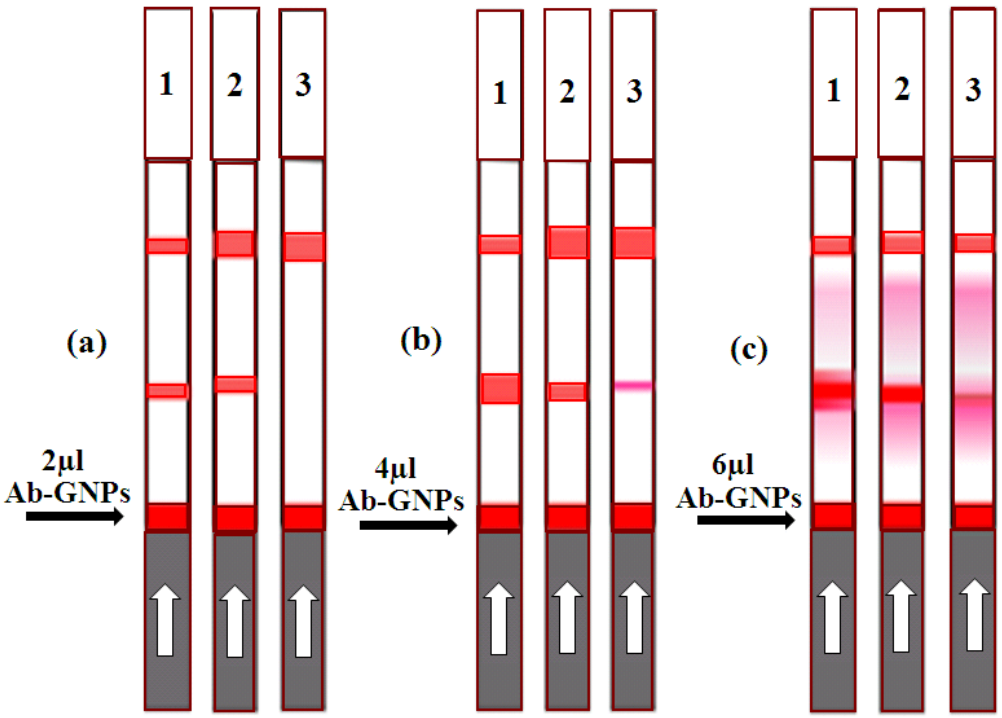

Figure 3. Illustration of the signals of loading 2, 4 and 6 µL of gold-labeled antibody for developing IgAD-ICG test strip. Every strip was tested with different concentrations of standard IgA (Lane 1: 3×10^4 ng/mL, Lane 2: 5×10^4 ng/mL, Lane 3: 30×10^4 ng/mL) to evaluate positive (Lane 1, 2) and negative (Lane 3) responses. Diagram (b) depicts the optimum.

coated on the nitrocellulose membrane as test and control lines. The proper amount of IgA antigen for the assay was determined at 2 µg/µL. To optimize the amount of gold-labeled antibody probe for the preparation of conjugated pad, different volumes of pAb-gold NPs (2, 4, and 6µL/strip) were examined. The results suggested that the optimal amount of colloidal gold–pAb was 4 µL/strip (Figure 3).

In the next step, the sensitivity and specificity of the ICG test strip were assessed. The sensitivity of the assay was investigated by testing IgA standard samples. The standards were prepared by the dilution of the IgA stock solution (1 mg/ml) with a IgAD serum sample to the final concentrations of 270, 90, 30, 5, 3, 1, and 0 ng/mL. Then, these standard samples were examined and judged by naked eye (Figure 4).

Serum samples containing IgA above 5×10^4 ng/mL presented a remarkable color on the control line but did not show the same color density on the test line.

Serum samples containing IgA less than 5×10^4 ng/mL presented a visible color on the test and control lines. These results showed that the detection limit of the test strip for monitoring human IgA in serum was 5×10^4 ng/mL (Figure 5).

To evaluate the specificity of the test strips, we investigated the potential cross-reactivity with serum proteins, IgG, IgM and human serum albumin in the same manner mentioned in IgA standard preparation.

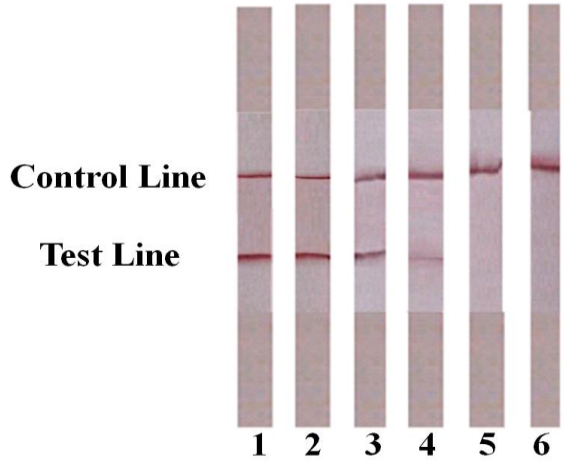

Figure 4. Test results of working standards with different concentrations of purified human IgA in serum using the strips. Lane 1: 0 ng/mL, lane 2: 3 ng/mL, lane 3: 5 ng/mL, lane 4: 30 ng/mL, lane 5: 90 ng/mL and lane 6: 270 ng/mL. The test was run three times at room temperature using IgA standard. Detection limit of the ICG test strip was determined at 5 ng/mL. The constant dilution ratio of 1:10,000 was used for assay. Serum samples should be diluted prior to testing.

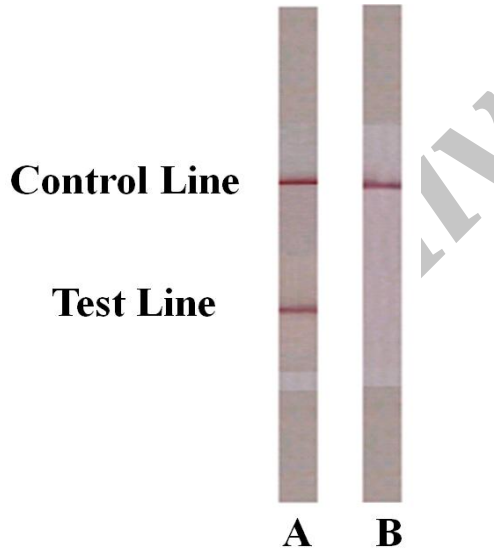

Figure 5. Typical results of the ICG test strip for IgA deficiency (competitive format). (A) Positive and (B) negative responses. Serum samples were diluted (dilution ratio 1:10,000).

The ICG test strips showed negative response (two clear red lines in the test and control lines) in the different concentrations of the compounds. The potentially cross-reactant proteins were added to the serum well above the maximum concentrations that will be encountered in clinical samples. The results were revealed that the ICG test strip had a high specificity to IgA without cross-reaction for other compounds.

The protocol of Omidfar et al.[28,30] was followed to test the stability of the presented ICG test strips. The results showed that the strips kept its stability for at least one month after storage at 45 °C and at room temperature for one year.

Comparison of the IgAD-ICG Test Strip with the ELISA

The reliability of the test strip was also examined by carrying out the ICG strip test with the 11 serum samples and compared the results with those obtained by ELISA (Table 1). The results revealed that our method and ELISA had significant concordance with each other in diagnostic judgment. These findings suggest that the ICG strip assay is simple, reliable and highly specific for rapid screening of the IgA-deficient patients.

DISCUSSION

Several methods for detection of IgA deficiency have been described until now including radial immunodiffusion, enzyme-linked immunosorbent assay (ELISA), solid-phase red cell adherence assay, membrane enzyme immunoassay (EIA), and the particle gel immunoassay.[31] Although these methods offer the advantages of high sensitivity and specificity, they are time-consuming, need experienced staff and should be performed in multiple steps. The utilization of these immunoassays has been confined to the equipped laboratories with tools and devices for performing the analyses. Latex agglutination inhibition test have been also developed for screening of IgA deficiency patients. However, it was a preliminary study and could not be used as an onsite diagnostic tool.[32,33]

In the present study, a novel inexpensive ICG rapid test was established successfully with a colloidal gold-IgA-specific pAb conjugate. This ICG assay is an easy, fast, semi quantitative, and competitive binding immunoassay for detecting IgA in serum in the range of 1-270 ng/mL. The method uses an alpha chain specific pAb to recognize human IgA selectively in test samples with a high degree of sensitivity and

specificity for monitoring of IgA in sera of the IgA deficient patients. In this assay, 100 µL of standard solution or sample was applied to the sample pad and then allowed to migrate up the membrane. As the reaction mixture reaches the test line containing purified human IgA, a competition occurs between the free IgA in the sample and the solid-phase IgA for binding to gold-anti-IgA probe. Since the sample solution reaches the control line on which rabbit anti-goat secondary antibodies were immobilized, any non-specific interaction can be trapped. At low concentration of IgA in the sample, the pAb-gold NPs will be attached to the test line to produce a red-colored band on the membrane. As the concentration of the IgA in the serum increases, more pAb-gold NPs conjugated to IgA pass through the first band (test line) and are attached to the second band (control line). The color signal could be read within 10 minutes. The positive result will be judged by the presence of two red lines both in the test and control lines. The test is assumed to be positive (IgA deficiency) since the color intensity of the test line is darker than or equal to the control line, and vice versa.

In this study, to develop a sensitive ICG assay, different volumes of gold NPs-conjugated antibody and also different concentrations of IgA were examined. We optimized 2µg/µL of IgA and 4 µL/strip of colloidal gold pAb, to detect the IgA with lower than 5×10^4 ng/mL in the serum sample of IgA-deficient patients. The specificity of ICG strip test was evaluated with other immunoglobulin and protein compounds. Our findings indicate that the developed ICG test strip has high specificity for human serum IgA and no reaction is observed with other compounds. The reliability of the test was examined by carrying out the ICG test strip with the serum samples obtained from 11 volunteers and the results from these tests were compared with those obtained by ELISA. It was found that the ICG test strip is applicable to detect IgA deficiency and could be used in screening programs.

ACKNOWLEDGEMENTS

This work was supported by Grants from Iran National Science Foundation (91004118) and Head & Neck Surgery Research Center of Iran University of Medical Sciences.

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