

Research Paper

Development of a sandwich enzyme-linked immunosorbent assay (ELISA) for determining of bovine serum albumin (BSA) in trivalent measles-mump-rubella (MMR) vaccines

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Abbreviations: BSA, bovine serum albumin; MMR, measles-mump-rubella; FCS, foetal calf serum; CCE, counter current electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; TMB, 3,3',5,5'-tetramethylbenzidine; HRP, horseradish peroxidase

Key words: BSA, MMR vaccine, polyclonal antibody, viral vaccine, sandwich ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA), using polyclonal antibody, was developed and compared with the commercial kit for detecting and estimating of BSA content in Measles-Mump-Rubella (MMR) vaccine samples in detection limit of nanogram level. The test depends on the capturing and detecting of BSA antigen by the polyclonal antibody. Initially, a detection range of 0–64 ng/ml was established, could be used for estimation of BSA content according to WHO requirement (50 ng/ml) in MMR vaccines. Comparative analysis of the test results for 85 MMR vaccine samples obtained with the commercial kit gave a sensitivity of 58.8% and a specificity of 97%. A high correlation ($r = 0.94$) was observed between BSA sandwich ELISA and commercial kit for BSA content in MMR samples. However, variations in values also were observed for the two assays. These variations may have been due to difference of upper limit of detection range of BSA content in commercial kit (32 ng/ml) and new sandwich ELISA (64 ng/ml) as well as the use of a different polyclonal antibody. In concerning the cutoff value for the WHO requirement and employment of standard solution of 64 ng/ml in developing assay, it would be adequate to use this test for assessing BSA content in viral vaccines same as MMR vaccines.

Introduction

The production of many viral vaccines in cell culture is still dependent on the presence of bovine serum in the cell culture medium.^{1,2} In order to avoid unwanted side effects after vaccination that might result from non-relevant proteins including bovine serum albumin (BSA) which is major protein present in bovine serum, should be kept to a minimum level.¹ Among viral vaccines, trivalent

Measles-Mump-Rubella (MMR) vaccine is produced in embryonated chicken cells in medium supplemented with foetal calf serum (FCS). As a consequence, all MMR vaccines contain varying amounts of residual BSA, depending on the specific manufacturing process. According to WHO requirements,³ the amounts of BSA in MMR vaccines should not exceed 50 ng/ml of vaccine.

Various techniques have been applied for estimation of bovine serum in the viral vaccines. Total amount of bovine serum proteins may be estimated by counter current electrophoresis (CCE) by using a rabbit antiserum directed against BSA. This method is time consuming and hardly applicable to large numbers of samples.^{1,4} An enzyme-linked immunosorbent assay (ELISA) which uses an anti-BSA monoclonal antibody was developed for detection of BSA with cutoff value of 60 ng/ml in live attenuated human rubella and measles vaccines.¹ A double sandwich ELISA with polyclonal antibody has also been developed for determination of FCS in MMR vaccines, monovalent measles vaccines, and so on.⁵

In the present work, we have developed a polyclonal antibody-based sandwich ELISA for determination of BSA in the MMR vaccines and its comparative efficacy with commercially available kit. The main advantage of the sandwich ELISA over double sandwich ELISA is the ability of this system for specific detection of BSA compared with FCS content of vaccines. The other advantage is, a shorter turnaround time because the developing assay is a direct sandwich ELISA.

Results

Characterization of anti-BSA antibody. Polyclonal antibody against BSA was raised in rabbits as described in the Materials and Methods. Characterization of antibody showed that antibody had specificity to its own antigen with minimal cross-reactivity to the control antigens. The ratios of specificity measured by direct solid-phase ELISA for anti-BSA were 3.507 (BSA/ovalbumin, BSA/horse serum) and 3.092 (BSA/human serum) respectively. These data obtained according to the following formula: OD of tested conjugate antibody to BSA antigen/ OD of tested conjugate antibody to control antigens.

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Optimal dilutions of reagents for BSA sandwich ELISA. The main objective of this study was to develop BSA sandwich ELISA that could detect BSA content of vaccines in nanogram quantities. The ability of polyclonal antibodies to capture or detect of vaccine containing BSA depends not only on the specificity of binding to BSA antigen but also on the relative amounts of these reagents in the assay. It was thus important to titrate both these critical reagents to determine the combination that yielded the best discrimination between 64 and 0 ng/ml of BSA standards. Optimal dilution of capture antibody was 1:8000 (Fig. 1A), while the preferred conjugate dilution was 1:100 (Fig. 1B).

Sandwich ELISA for BSA quantification. To evaluate the amounts of BSA in vaccine samples, we used standard solutions which were prepared as described earlier. Standards were applied in every plate the same way as the samples. Standard curve was established for BSA (Fig. 2).

Comparison of commercial sandwich ELISA kit and BSA sandwich ELISA. A comparative analysis of the commercial sandwich ELISA kit and the BSA sandwich ELISA was carried out to establish diagnostic parameters like sensitivity and specificity and to study the distribution of BSA content values obtained by both assays. Regression analysis of results on these samples revealed a strong positive correlation between BSA contents ($r = 0.94$) (Fig. 3) in two systems. The results of both assays for 85 vaccine samples indicated that 14.11% of them were positive by the BSA sandwich ELISA, while 20% of the samples were positive by use of the commercial kit according to the WHO requirement.

The sensitivity and specificity of the system described here versus the commercial kit are 58.8% and 97% respectively. The data for the above analysis are shown in Table 1.

Discussion

Residual bovine serum proteins in viral vaccines are a well-known problem in vaccine production.⁵⁻⁷ For some vaccines upper limits for the content of these proteins have been established or recommended by WHO, e.g., 50 ng/ml of BSA content for live attenuated MMR vaccine.³ The bovine serum content of viral vaccines can be reduced by additional purification steps.^{5,7} Since all purification methods also lead to losses of viral antigens, evaluation of the effect of different measures to reduce the amount of bovine serum during culture and purification is important. The bovine serum content of current vaccines is generally low and this protein cannot usually be detected in final vaccines.

The availability of a simple, fast, reliable and inexpensive test would provide a tool that can be readily used for detection and estimation of residual bovine serum proteins. BSA sandwich ELISA described in the present work fits that role. This assay was used for BSA detection as the major protein of bovine serum in level of ng/ml of MMR vaccine samples.

The polyclonal antibody (capture and conjugate) used for the BSA sandwich ELISA was raised against BSA and was specific to it. This polyclonal antibody appeared to be unaffected by the control antigens.

The optimal dilution of capture and conjugate selected were 1:8000 and 1:100, respectively (Fig. 1). These dilutions obtained by the dilution of capture antibody was increased, but conjugate remained (1:100) (Fig. 1A). The capture antibody dilution remained

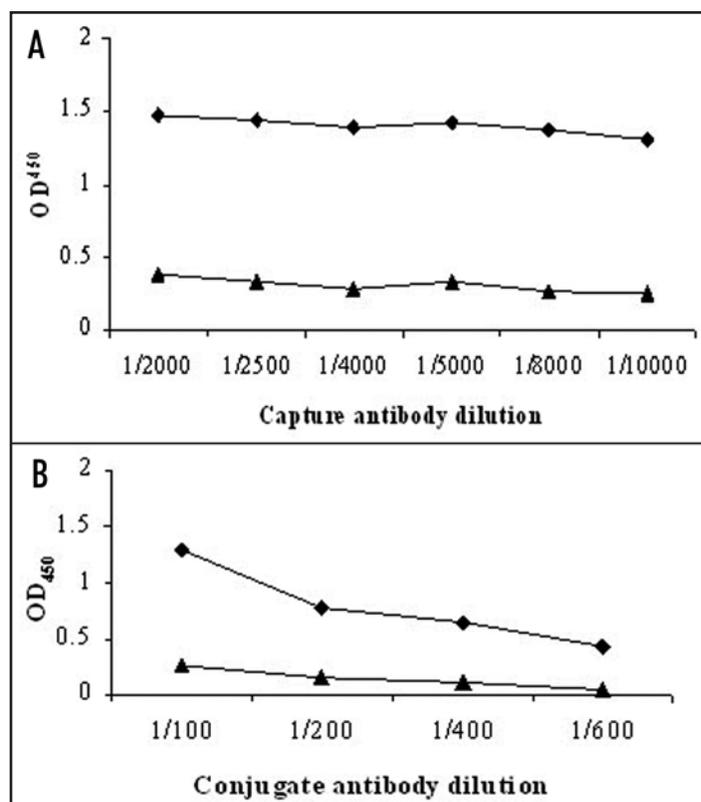


Figure 1. (A and B) Determination of the optimal dilution of capture antibody and conjugate antibody for bovine serum albumin (BSA) sandwich enzyme-linked immunosorbent assay (ELISA). (A) Different dilutions of capture antibody reacted with negative (solid triangles) and positive (solid diamonds) standard solutions against 1:100 dilution of conjugate antibody. A capture antibody dilution of 1:8000 was selected. (B) A 1:8000 dilution of capture antibody reacted with the same standards versus various dilutions of conjugate antibody; a dilution of 1:100 for conjugate antibody was selected.

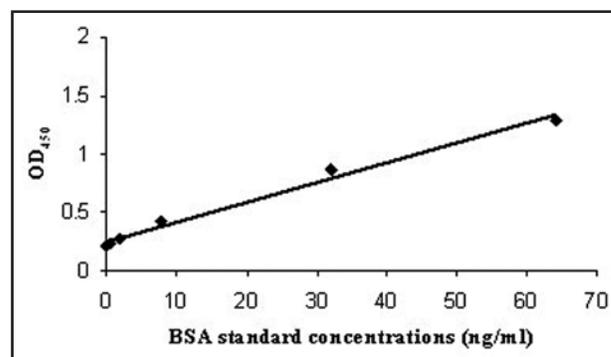


Figure 2. Standard curve for BSA sandwich ELISA.

(1:8000), but the conjugate was diluted; the differences in OD can be seen in Figure 1B.

The results obtained by the BSA sandwich ELISA, which uses a detection range of 0–64 ng/ml for BSA content, and the reference kit, which uses detection range of 0–32 ng/ml are comparable. A good correlation ($r = 0.94$) in terms of efficiency was found between the BSA sandwich ELISA and commercial kit (Fig. 3). According to WHO requirement maximum BSA content in MMR

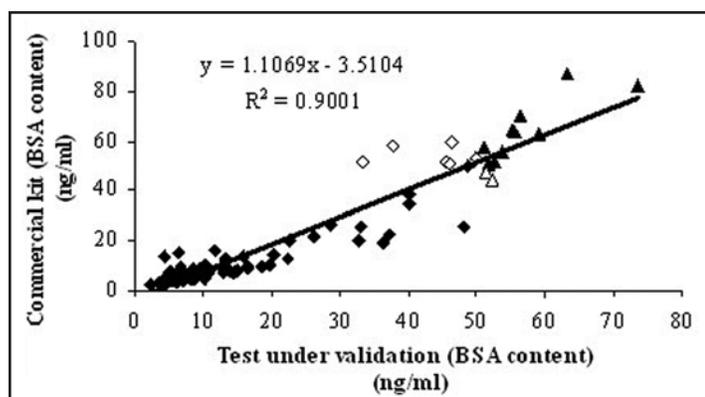


Figure 3. Comparative efficacy of the BSA sandwich ELISA, developed for the detection of BSA contents in MMR vaccine samples (N = 85). Figure is based on sample regression analysis. Black solid diamonds, Negative samples for BSA content in commercial kit and new ELISA assay; Black solid triangles, Positive samples for BSA content in commercial kit and new ELISA assay; White solid diamonds, Negative samples for BSA content in new assay and positive in commercial kit; White solid triangles, Positive samples for BSA content in new assay and negative in commercial kit.

Table 1 Two-sided contingency table for comparative efficacy of BSA sandwich ELISA on 85 MMR vaccine samples tested in parallel using both the systems (commercial kit and the BSA sandwich ELISA)

Total	Commercial kit			BSA sandwich ELISA
	Negative	Positive		
12	2	10	Positive	
73	66	7	Negative	
85	68	17	Total	

vaccines is 50 ng/ml thereby we used a standard solution of 64 ng/ml as an upper limit. This ELISA showed a sensitivity of 58.8% and a specificity of 97% by considering the upper limit of 64 ng/ml but these data for the sandwich ELISA with respect to upper limit of 32 ng/ml were 100% and 92.06% respectively. Our ELISA detected 5.88% more negative samples than the commercial kit with respect to detection limit of 50 ng/ml. Commercial kit detected 5.88% more positive samples according to upper limit of 32 ng/ml. Specific detection of recently assay possibly for definite detection limit of 32 to 64 ng/ml.

In conclusion, BSA sandwich ELISA developed in this work can be used for BSA quantization purposes and might help to detect specific BSA content in viral vaccines same as MMR vaccine samples.

Materials and Methods

Antigens. BSA (Biochemica, Fluka) was used to produce polyclonal antibody and developing a sandwich ELISA. To detect the specificity of the test, ovalbumin (Sigma), horse and human sera (Biotechnology Department, Razi Vaccine and Serum Research Institute) were used as the control antigens in a direct ELISA.

Preparation of BSA standards. The standards were prepared by weighing and dissolving of 64 mg of BSA in 1 ml of Dulbecco

Modified Eagle's Medium (DMEM) (Sigma). From this solution as a stock solution, the necessary standards 64, 32, 8, 2 and 0.5 ng/ml were made. DMEM without BSA was used as the standard solution of 0 ng/ml.

Test samples. A total of 85 MMR vaccine samples (From one batch) were obtained from human viral vaccine department of Razi Vaccine and Serum Research Institute in Iran; this live attenuated vaccine had been produced in chicken embryo cells in DMEM supplemented with FCS. All the samples were frozen at -20°C until use.

Preparation of polyclonal antibody. Polyclonal antibody against BSA was raised in rabbits by immunizing subcutaneously with 1 mg/weight of BSA in complete Freund's adjuvant. Boosters were performed twice with BSA (0.5 mg of protein/kg), mixed by noncomplete Freund's adjuvant. Bleeding was performed on days 37 to 40 and sera were isolated. Anti-BSA γ -globulins were precipitated with 35% ammonium sulfate, suspended in 1/10 volume of 0.01 M phosphate buffered saline (PBS) and dialyzed against 0.9% saline three times over 24 h. The specificity of precipitated antibody was evaluated by direct ELISA (see procedure below) with control antigens.

Reference assay kit. A BSA sandwich ELISA (Cygnus Technologies, USA) was used as the reference kit. The standards in the reference kit were from 32 to 0 ng/ml (32, 8, 2, 0.5 and 0 ng/ml).

Sensitivity and specificity of the BSA sandwich ELISA. To calculate the sensitivity and specificity of the BSA sandwich ELISA, the samples were tested with the developed assay and commercial kit. The results obtained with the commercial kit, which were considered as the "golden standard" and the following formulas were used to determine the sensitivity and specificity of the BSA sandwich ELISA: sensitivity = (number of samples with positive results by BSA sandwich ELISA and commercial kit/number of samples with positive results by BSA sandwich ELISA but positive by commercial kit plus this number) \times 100, and specificity = (number of samples with negative results by BSA sandwich ELISA and commercial kit/number of samples with positive results by BSA sandwich ELISA but negative by commercial kit plus this number) \times 100.

Direct ELISA. Direct ELISA was performed to determine the cross reactivity of polyclonal antibody. Briefly, the wells in a 96-well plate (NUNC, Denmark) were coated with BSA and control antigens (ovalbumin, human serum and horse seum) at 100 μL /well containing 320 ng in carbonate bicarbonate buffer, pH 9.6, and were incubated overnight at 4°C . The excess protein was washed out with PBS containing 0.05% Tween 20 (PBST). The plates were blocked with blocking buffer, probed with an appropriate dilution of the tested antibody conjugated with horseradish peroxidase (HRP) for 1 h at 37°C and washed five times with PBST. The optimal dilution of conjugate, 1:100 was determined by direct ELISA for BSA antigen. The reaction was developed by using 3,3',5,5'-tetramethylbenzidine (TMB). Finally the reaction was stopped using 1 N H_2SO_4 and the wells were read at 450 nm in an ELISA reader.

Sandwich ELISA. Polystyrene 96-well plates were coated with 100 μL /well of anti-BSA γ -globulin (capture antibody) in carbonate bicarbonate buffer, pH 9.6, at 4°C overnight. The plates were rinsed four times with PBST and blocked with blocking solution at 37°C for 1 h. After being washed four times, the following reagents were added in a single step and incubated for one hour at 37°C : first, 100 μL /well of test sample or standard solution. Secondly, 100 μL

of anti-BSA γ -globulin-HRP conjugate at 1:100 dilution. After five washes, bound HRP conjugate was detected with TMB for 20 min (100 μ L/well) at room temperature. The reaction was terminated using 1 N H_2SO_4 (50 μ L/well), and the absorbance was measured at 450 nm.

Determination of optimal capture antibody and conjugate dilutions. Two BSA standards (one 64 ng/ml and one 0 ng/ml) were chosen for use in the establishment of BSA sandwich ELISA parameters. For establishment of the optimal dilution of the capture antibody, the anti-BSA γ -globulin-HRP conjugate was tested at a fixed dilution of 1:100 against different dilution of capture antibody. Similarly, the optimal conjugate dilution was determined by testing of various dilutions of the conjugate against the optimal capture antibody dilution. The optimal dilution of each reagent was determined to be the maximum differential in OD values between positive (64 ng/ml) and negative (0 ng/ml) standards.

Comparison of BSA sandwich ELISA and reference kit. The 85 vaccine samples were tested for the BSA content by using both the reference kit and the newly developed sandwich ELISA. The results of both assays were read to determine whether each sample was positive or negative (upper limit: 32 ng/ml for the reference kit, 64 ng/ml for the BSA sandwich ELISA, while detection limit for BSA content of MMR vaccines was 50 ng/ml).

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