Talanta 82 (2010) 432–436

Contents lists available at ScienceDirect

Talanta

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

Short communication

A simple and rapid competitive enzyme-linked immunosorbent assay (cELISA) for high-throughput measurement of secretory immunoglobulin A (sIgA) in saliva

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ARTICLE INFO

Article history:
Received 25 February 2010
Received in revised form 13 April 2010
Accepted 16 April 2010
Available online 22 April 2010

Keywords:
Immunoglobulin A (IgA)
Saliva sample
Competitive enzyme-linked immunosorbent assay (cELISA)

ABSTRACT

A simple competitive enzyme-linked immunosorbent assay (cELISA) was established for rapid measurement of secretory immunoglobulin A (sIgA) in saliva. The method was based on competitive reaction between the immobilized IgA and free IgA in the solution for the limited amount of horseradish peroxidase-conjugated rabbit anti-human IgA. In comparison with the conventionally used Sandwich ELISA, the cELISA is simpler, low-cost, and shows better reproducibility since it is not affected by the variation of capture antibodies from different batches. The assay time was also significantly reduced from more than 5 h to less than 3 h. Different curve-fitting models were compared, among which the fully specified logit-log model gave the best results. The linear working range and limit of detection were found to be 0.1–100 $\mu g\,mL^{-1}$ and 0.05 $\mu g\,mL^{-1}$, respectively. Matrix effects of saliva samples were investigated and a reasonable range of dilution factors were proposed. The developed method offers a very practical approach for high-throughput measurement of sIgA in saliva samples.

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1. Introduction

Secretory immunoglobulin A (sIgA) is the most important antibody in mucosal immunity [1–3]. It helps to fight against the ingested, inhaled or body surface-contacted pathogens. In recent years, particular interest has been directed toward evaluating the potential effect of environmental toxicants on the immune system of residents, especially children [4]. Though serum has been widely used for immunological diagnosis [5], it is invasive and not suitable for epidemic studies with a relatively large population. The adoption of saliva samples offers a good alternative to overcome these difficulties [6]. So, simple and rapid ways for quantification of sIgA in saliva are of great value for high-throughput analysis of such large number of samples.

sIgA has been previously detected by single radial immunodiffusion technique on agar gel layers [5–8]. But this technique takes very long time (about 48 h) and the sensitivity is low. Sandwich enzyme-linked immunosorbent assay (ELISA) and immunoelectrophoresis are also developed for the measurement of salivary sIgA [9,10]. Sandwich ELISA is the most commonly used ELISA mode for detection of large antigen molecules. However, it relies on two types of antibodies that recognize different epitopes on the analyte. Careful consideration is often required about which antibody to be used as the label and which as the capture ligands since the binding of one antibody to the antigen may increase or decrease the affinity of binding of the second antibody [11]. In our preliminary experiments with the Sandwich ELISA, we encountered several problems. First, the assay time was very long. Starting from the addition of the tested samples, it took more than 5 h to complete the total measurement, mainly due to the two stages of immunoreaction. Second, the calibration curve varied remarkably with different batches of the capture antibodies. In addition, non-specific binding between the two antibodies was observed from time to time which showed substantial influences on the measurement results.

To address these issues, we turned to the competitive modes since they only need one type of antibody. In competitive ELISA (cELISA), either the antibody is coated on the plate, to which the free analyte antigen and the labeled antigen are added or the antigen is immobilized, to which the free analyte antigen and the labeled antibody are added. Since it is generally easier to obtain the enzyme–antibody conjugates (either the antibody is directly labeled with enzyme or it is further detected by the enzyme-labeled second antibody) than the labeled antigen, a cELISA with immobilized antigen can be more readily established for many analytes of interest [12]. So in this work, we attempt to develop a cELISA with immobilized antigen and labeled antibody for salivary IgA detection, mainly to reduce the time and reagent cost and also to improve the reproducibility of the method. The curve-fitting method for the data manipulation and guidelines for choosing reasonable dilution times for the real samples were also investigated in detail. The advantages of the presented method were demonstrated by

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doi:10.1016/j.talanta.2010.04.040

the successful application to analyze sIgA in twenty children saliva samples.

2. Experimental

2.1. Reagents and instrumentation

Human immunoglobulin A (IgA) and horseradish peroxidase (HRP)-conjugated rabbit anti-human IgA were purchased from Bioss tech., China. 5,5′-tetramethylbenzidine (TMB) was purchased from Sigma Chemical Co. (USA). A Human IgA ELISA Quantitation Kit based on Sandwich ELISA was obtained from Bethyl Laboratories (Montgometry, USA) and used for comparison with the established method. All other chemicals used in this study were of analytical reagent grade or better. Microwell plates were purchased from Nunc, Denmark and a Tecan Genios Microplate Reader (Austria) was used to measure the optical density of the ELISA results, with the absorbance and reference wavelengths at 450 and 492 nm, respectively. An Avanti J-25 high-speed refrigerated centrifuge from Beckman (USA) was used for centrifugation.

2.2. Competitive enzyme-linked immunosorbent assay (cELISA)

100 μL of 2.5 μg mL^{-1} human IgA in 0.05 mol L^{-1} carbonate buffered saline (pH 9.6) was coated onto the 96-well microplate at 4 °C overnight. The plate was washed with 0.01 mol L^{-1} phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) for three times. Then the wells were blocked with 1% gelatin in 0.01 mol L^{-1} PBS at 37 °C for 1.5 h, followed by washing with PBST for three times. Next, 50 μL of IgA standard solutions or diluted saliva samples were added to the wells, followed by addition of 50 μL of 1.5 μg mL^{-1} HRP-conjugated rabbit anti-human IgA. The plate was incubated at 37 °C for 1.0 h under shaking at 100 rpm. Then the plate was washed by PBST for three times and water for twice, and 100 μL of TMB solution containing H_2O_2 (30%), TMB (6 mg mL^{-1}) and phosphate buffer (0.1 mol L^{-1}, pH 6.0) in a volume proportion of 1.5:10:1000 was added to the wells for color development. After 15 min, 50 μL 2 mol/L H_2SO_4 was added to stop the reaction. The absorption was read at 450 nm with the microplate reader. The experimental conditions were optimized for each of above steps.

2.3. Comparison of different curve-fitting methods

Four commonly used mathematical models (log *A*–log *c*, four-parameter logistic-log, the partially specified logit-log and the fully specified logit-log models) are compared for establishing the calibration curves for the cELISA.

The four-parameter logistic equation [13–15] is described as:

$$y = \frac{a-d}{1+(x/c)^b} + d$$

where y is the response, x is the analyte concentration, a and d are the responses at zero and infinite concentrations, respectively. b is the slope factor and c is the ED_{50} (concentration at the true midpoint of the curve).

Logit-log model has two forms, the fully specified logit-log model is described as

$$\log\left(\frac{A-A_0}{A_m-A}\right) = a + b\log c$$

and the partially specified logit-log model is described as:

$$\log\left(\frac{A}{A_m-A}\right) = a + b\log c$$

where a and b are the y intercept and slope for the line, respectively. A_0 and A_m are the ODs at zero and infinite concentrations, respectively.

2.4. Saliva sample collection and optimization of the dilution times

Whole unstimulated saliva samples (0.5 mL) were collected from primary school students 10–12 years old during the lunch break time. All the participants had their regular lunch and rinsed their teeth with pure water at least 30 min before the collection. The samples were centrifuged at 10,000 rpm for 25 min at 4 °C. The supernatant was separated and diluted with 0.01 mol L^{-1} PBS before analyzed by cELISA. Different dilution times in the range from 5 to 400 folds were tested and evaluated, based on which the optimum dilution times was determined.

2.5. Quantitative comparison with the Sandwich ELISA Test Kit

The Sandwich ELISA Test Kit was used following the manufacturer's instructions with some modifications. The reaction time and the sample dilution folds were both optimized. Twenty children saliva samples were collected and measured with both the established cELISA method and the Sandwich ELISA Test Kit under their respective optimum experimental conditions.

3. Results and discussion

3.1. Optimization of the experimental conditions for cELISA

In the cELISA, the concentrations of the coated antigen and the enzyme-labeled antibodies were first optimized by chequerboard titration (see Fig. S1 in the Supporting Information). The optimum concentrations were found to be 2.5 μg mL^{-1} for human IgA and 1.5 μg mL^{-1} for HRP-conjugated rabbit anti-human IgA, respectively. In the test with different blocking reagents, gelatin can prevent the nonspecific reaction more efficiently than bovine serum albumin (BSA) or the skim milk powder. No significant differences were observed when the competitive reaction was conducted at pH 7.4 or 8.0. The addition sequence of the free IgA and the enzyme-labeled antibody was found to have substantial influences on the reaction time. In the case with small molecules, free antigen is usually mixed with the antibody first, then the solution was added to the well with immobilized antigen to avoid competitive discrimination. But in the present case, better results were obtained when free IgA solution was added first, followed by addition of HRP labeled anti-IgA antibody. As shown in Fig. 1a, the absorbance remains unchanged between 1 and 2 h, indicating that the reaction is already stabilized after 1 h. But in Fig. 1b, the reaction is far from completeness after 1 h. This is most probably because the free antigen and bound antigen are of the same size and property. When they are premixed before the addition of the antibody, the competitive is very effective and the reaction quickly reaches equilibrium (≤1 h). Contrarily, premixing of the IgA standard solution or the saliva sample with the labeled antibody before addition to the wells resulted in very slow reaction rate (incubation time ≥2 h to get to equilibrium) (Fig. 1b). This is reasonable since most of the antibody has formed complex with the free IgA in the premixing solution, it is much more difficult for the immobilized IgA to replace the free IgA in the antibody–antigen complex. Furthermore, the regular decrease of absorbance in Fig. 1a also offers a good accuracy and sensitivity of the sequential competitive test. Under the optimum experimental conditions, the total assay time of the cELISA is less than 3 h, which is only about half of the Sandwich mode.

3.2. Comparison of different fitting models

Standard curves of the cELISA were generated by using the four mathematical models (log *A*–log *c*, four-parameter logistic-log, the

Fig. 1. Influence of the incubation time and the concentration of free IgA with the enzyme-labeled antibody on the competitive reaction with fixed IgA of the cELISA. (a) Free IgA solution was added first, followed by addition of HRP labeled anti-IgA antibody; (b) premixing the IgA standard solution with the labeled antibody before addition to the wells.

partially specified logit-log and the fully specified logit-log models). The results of the four models were compared in Fig. 2. As can be seen, the log A–log c model shows a good linear fitting only in the high concentrations range. The four-parameter logistic model shows an S-shaped curve with very good R^2 (0.9995). However, the linear range of the curve is very narrow, which is also not suitable for practical use. Then the data were further analyzed using the two log-logit transformations.

The linear regression equations of the partially specified logit-log and the fully specified logit-log models are found to be $\log(A/(A_m - A)) = 0.687 - 0.990 \log c$ (μg mL^{-1}) ($R^2 = 0.991$) and $\log((A - A_0)/(A_m - A)) = 0.792 - 1.234 \log c$($\mu$g mL^{-1}) ($R^2 = 0.997$), respectively. As can be seen from the two lines shown in Fig. 2c, the two logit-log functions both achieved good linearity in the tested concentration range. Compared with the partially specified logit-log model, the fully specified logit-log transformation performed better over the low concentration range (standards within 15% of the fitted line). This should be attributed to the precise specification of two unknown quantities, the optical densities at zero and infinite concentrations, prior to fitting the model to a typical set of calibration data. This model also has the advantage that it is easiest to visualize since it does not incorporate complex transformations of the optical density scale. Partially specified logit-log model can be regarded as a reduced form of the fully specified logit-log model

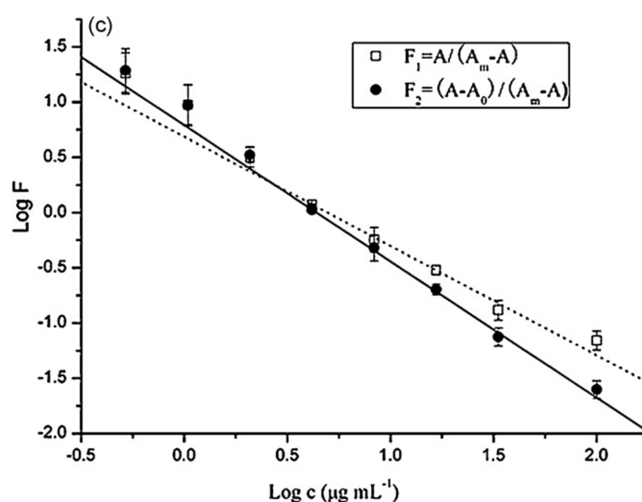

Fig. 2. The fitting curves of the competitive ELISA using (a) log A–log c model. (b) Four-parameter logistic model, (c) the partially specified logit-log (dotted line) and the fully specified logit-log models (solid line).

derived by assuming that the OD corresponding to a zero concentration should be 0, resulting in the A_0 parameter being set equal to 0. But in this system, the incorporation of the A_0 is important. So the fully specified logit-log function was chosen for performing standard curves and for interpolating IgA concentrations from the standard curve in the following experiments.

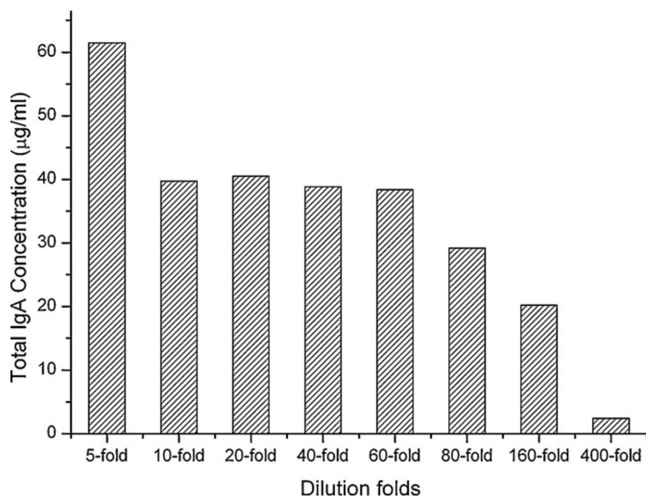

Fig. 3. Influence of the dilution factor of saliva on the measurement of the IgA concentration by cELISA.

The linear working range of the cELISA method is found to be 0.1–100 $\mu g\,mL^{-1}$, with the limit of detection of 0.05 $\mu g\,mL^{-1}$ (defined as three times the standard deviation of the blank). Since no capture antibody was used in the competitive mode, the assay was less affected by the variations of antibodies from different batches (see Fig. S2 in the Supporting Information). For comparison, the effects of using different batches of capture antibodies on the results of Sandwich ELISA were also shown in Supporting Information (Fig. S3). The intra-assay and inter-assay coefficients of variation of the cELISA were found to be in the range of 3–5% (calculated from testing the same sample in quadruplicate in one assay) and 5–10% (determined by measurement of the same sample in separate assays, n = 4), respectively.

3.3. Optimization of the dilution times in the detection of real saliva samples

A fundamental problem with the analysis of components in biological samples is the effect of the extremely complex and variable mixture of proteins, carbohydrates, lipids, small molecules and salts constituting the sample [16]. Morgan et al. [17] reported that the viscosity of the sample might be an important interference of matrix effect. As a mixture of salivary-gland secretion and gingival crevicular fluid, the saliva samples are quite sticky with complex matrix components. Due to the high specificity and sensitivity of immunoassays, dilution has been commonly used to reduce the matrix effect in ELISA [18,19]. Ideally, when a sample is serially diluted, the product of the measured concentration multiplied by the dilution factors should remain fairly constant for each dilution and closely approximate the original concentration. However, an arbitrary dilution of the real sample may often lead to false results, which is sometimes ignored in practical analysis. Fig. 3 shows the calculated IgA concentrations of a tested saliva sample with different dilution factors in the range from 5 to 400. Clearly, the appropriate dilution factors should be between 10 and 60. The 5-fold dilution was not sufficient to totally eliminate the matrix effect, so the IgA concentration was overestimated. On the other hand, when the dilution factors are too large (>80 in this case), the results are also unreliable. One of the major reasons for this is the final concentration of the diluted sample is close to or even lower than the lower limit of the calibration curve. Thus the error is also increased. Above optimized dilution range was further confirmed by other two saliva samples with different IgA concentrations. The samples were diluted at 20-, 50-, 100- and 200-fold, respectively, the corresponding total IgA concentrations of the first sample were found to be 62.7, 61.4, 60.3 and 45.1 $\mu g\,mL^{-1}$, respectively; while for the second sample, the calculated total IgA concentrations were 30.3, 34.7, 22.9 and 16.7 $\mu g\,mL^{-1}$, respectively. So a proper dilution should not only sufficiently reduce the matrix effect but also try to make the obtained concentration fall into the central portion of the linear working range. Otherwise, the calculated results of the original sample would be totally wrong.

The recovery of the method was checked by spiking two individual samples with known amount of IgA standard. The results are listed in Table 1.

Table 1
Recovery test results of the established cELISA (n = 4).

Sample	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Recovery (%)
A	0	21.4	112 ± 6
	6.7	28.9	
B	0	34.2	91 ± 3
	6.7	40.3	

3.4. Quantitative comparison with the Sandwich ELISA Test Kit

According to above experimental results, the reaction time and sample dilution factors were also optimized for the Sandwich ELISA Test Kit. After blocking the wells, the samples were diluted serially in the range from 100 to 1000 folds and incubated with the immobilized capture antibody for different time periods (40, 80, 120 and 160 min). As shown in Fig. S4 in the Supporting Information, the reaction between the sample and the capture antibody was quite slow and it took more than 2 h for the reaction to complete. The working range and proper dilution range of the Sandwich kit are determined to be 8.0–500 $ng\,mL^{-1}$ and 200–800 folds, respectively. A previous work by Miletic et al. [18] used 1:200 diluted saliva samples to determine the salivary IgA secretion rate in young and elderly persons by a Sandwich ELISA. This dilution factor is generally in agreement with the above-recommended range.

The two ELISA methods were used to analyze the collected children saliva samples under their respective optimum experimental conditions and the results are compared in Fig. 4.

A good correlation between the two methods can be seen from the data shown in Fig. 4. The linear correlation equation was c(cELISA, $\mu g\,mL^{-1}$) = 1.113 c(Sandwich Kit, $\mu g\,mL^{-1}$) + 0.519 with the correlation coefficient of 0.991 (n = 20). The cELISA shows obvi-

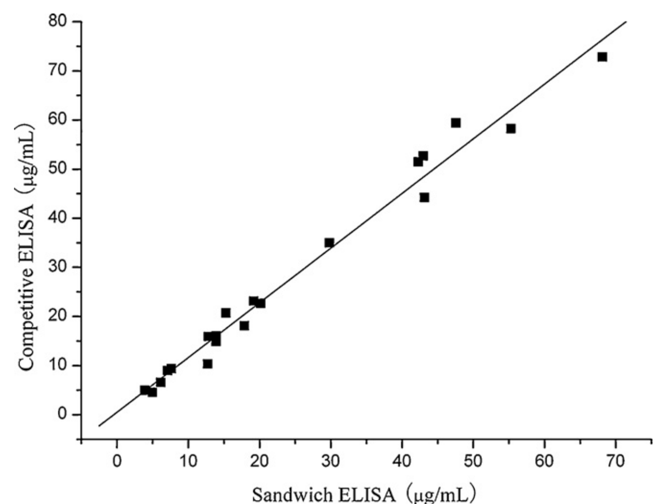

Fig. 4. Correlation between IgA concentrations of children saliva samples measured by cELISA and a classical Sandwich ELISA test.

ous advantages of simplicity, rapidity and low-cost in detection of large amount of salivary samples. Under our experimental conditions, the IgA concentrations of the tested children saliva are in the range of 3.9–68.1 $\mu g\,mL^{-1}$. According to previous reports, the IgA concentration in human saliva is related to the age, racial, health condition and other factors [5,9,20]. The sampling time and manner may also affect the measurement results. These factors should be carefully considered in planning research studies on the effect of environmental quality on the residents' health.

As a noninvasive diagnostic medium, saliva sample can be collected without medical supervision and allows frequent sampling. These practical advantages over blood are of great importance for epidemic study. The cELISA presented in this paper offers a simple and rapid way for quantification of IgA in large number of saliva samples. It will also be very helpful for developing bioassays for the detection of other analyte of interest with large molecular weight.

4. Conclusion

A competitive ELISA has been developed for human salivary IgA detection. In the method, the reaction time and reagent cost are greatly reduced and the reproducibility is improved. The linear working range of the method is from 0.1 to 100 $\mu g\,mL^{-1}$, which is suitable for analysis of real saliva samples. The fully specified logit-log function gives the most reliable measure of the IgA concentration. A proper range of sample dilution factors was also proposed. The assay can be performed within 3 h and the intra-assay variations were less than 5%. The developed method offers a good alternative of the classical ELISA test with a rapid, simple and low-cost analytical approach for high-throughput measurement of sIgA in saliva samples.

Acknowledgement

The work was supported by the National Natural Science Foundation of China (20637020).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.04.040.

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