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Development and Optimization of ELISA for Serum Samples of Bovine Origin

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ABSTRACT: The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical immunochemistry assay based on the specific bond between the antigen and the antibody. The application of this test has significantly changed the practice of animal laboratories in which it is used for detection and quantification of molecules such as hormones, peptides, antibodies, and proteins. Various technical variants of this test can detect antigen (native or foreign) or antibody, determine the intensity of the protein response whether pathological or not; the type of induced immune response as well as the innate immunity potential; and much more. These capabilities, as well as the high sensitivity and robustness of the test and a small price, make it quick and reliable diagnostic assay. Despite being used for nearly 50 years, a variety of ELISA tests with different technical solutions are still being developed, improving and extending the application of this outstanding test. Some challenges of the study are 1. Optimizing and standardizing enzyme immunoassays for veterinary applications may involve dealing with complex matrices in animal samples, which can introduce interfering substances affecting the assay's accuracy and specificity. 2. Validating the performance of ELISA tests for various veterinary molecules requires addressing issues related to cross-reactivity, sensitivity, and reproducibility, especially when dealing with diverse animal species and varying physiological conditions. 3. Developing and implementing new technical variants of ELISA while ensuring their reliability and comparability to existing methods pose challenges in terms of establishing appropriate reference standards and controls for accurate result interpretation. The purpose of this research article is optimization, standardization and validation of enzyme immunoassays in the veterinary field by overcoming all the challenges.

Keywords: ELISA, Optimization, Validation, Antigen and Antibody.

INTRODUCTION

The plate-based assay method known as ELISA (enzyme-linked immunosorbent assay) is used to identify and measure soluble molecules such as peptides, proteins, antibodies, and hormones. An ELISA involves immobilising the target macromolecule (antigen) on a solid surface (microplate) and then combining it with an antibody that is connected to a reporting enzyme followed by incubating the reporter enzyme with the proper substrate to produce a quantifiable end product. The highly specific antibody-antigen interaction is the most important component of an ELISA. With ELISA, a wide range of samples can be evaluated, and the assay conditions chosen will depend on the complexity of the sample and the anticipated concentration of analyte present.

Types of ELISA: The crucial procedure in the ELISA assay is adhering or immobilizing the antigen or antigen- specific capture antibody directly to the well surface in order to directly or indirectly detect the antigen. Using a "capture" antibody, the antigen can be precisely identified from a sample of mixed antigens for accurate and reliable assays. As a result, the antigen is "sandwiched" between the capture antibody and the detection antibody. If the antigen to be assessed is tiny or has just one epitope for antibody binding, a competitive technique is utilized in which either the antigen is labelled and competes for the formation of an unlabeled antigen-antibody complex or the antibody is labelled and competes for the bound antigen and antigen in the sample (Gan and Patel 2013).

Direct ELISA: A sample that needs to be tested for a particular antigen is immobilized to the wells of a micro titer plate, and any wells that are left uncoated with the antigen are blocked with a solution of a non-reacting protein, like bovine serum albumin. The secondary antibody that has been enzyme-conjugated is then added after the primary antibody, which binds precisely to the antigen. The enzyme's substrate is added in order to measure the primary antibody by altering color. Color intensity is directly correlated with the amount of primary antibody present in the serum. The fact that the

antigen immobilization technique is non-specific is a major drawback of indirect ELISA. When serum is employed as the test antigen, all sample proteins may stick to the wells of a micro titer plate (Gan and Patel 2013).

Sandwich ELISA: The sandwich method is employed to target a particular antigen present in sample. To capture the target antigen, the well surface is prepared with a specified quantity of capture antibody. Bovine serum albumin is used to inhibit non-specific binding sites before the antigen-containing sample is added to the plate. The antigen is then "sandwiched" by a particular primary antibody. Secondary antibodies that are enzyme-linked to the original antibody are used. Washing of any antibody-enzyme conjugates that are not bound is done. The addition of a substrate results in an enzymatic conversion that produces a quantifiable color (Gan and Patel 2013). In order to quantify human KGF, Canady *et al.* (2013) used the sandwich approach to analyze patient sera to find elevated keratinocyte growth factor (KGF) levels in the sera of keloid and scleroderma patients in comparison to healthy controls. Utilizing a purified particular antibody to capture antigen has the benefit of reducing the requirement to separate the antigen from a mixture of other antigens, simplifying the assay and enhancing its specificity and sensitivity (Gan and Patel 2013).

Competitive ELISA: The process of competitive interaction between the sample antigen and antigen bound to the wells of a micro titer plate with the primary antibody is the main event of competitive ELISA. The sample antigen and primary antibody are first incubated together, and the resulting antibody-antigen complexes are then introduced to wells that have already been coated with the same antigen. Any unbound antibody is rinsed away during an incubation period. More primary antibodies will bind to the sample antigen the more antigen is present in the sample. As a result, less primary antibody will be available to bind to the antigen coated on the well. A substrate and a secondary antibody that is enzyme-conjugated are then added to produce a chromogenic or fluorescent signal. Antigen is present in the sample if there is no color (Gan and Patel 2013). The primary benefit of competition ELISA is that it is highly sensitive to compositional variations in complicated antigen mixtures, even when the specific detecting antibody is present in only minor quantities (Dobrovolskaia *et al.*, 2006).

Even though this technique can be performed easily with great sensitivity, there exists certain problems at some levels which cannot be neglected while optimization and development of an ELISA. The development of an ELISA kit requires several reagents and constituents to be put in specific order and quantity so that at the end colour produced by the combination can be quantitatively interpreted to determine the concentration of the analyte in the sample. Success of an ELISA depends on optimization of these several factors. Since ELISA is a multi-step process, each stage can be independently tested before an experiment begins. Antigen or antibody coating, saturation, blocking, analyte application, detection with the proper antibodies (primary and secondary), and signal detection make up the ELISA technique. The definition of optimization is the empirical determination of the ideal concentrations of each assay reagent and the ideal circumstances for each stage (Minic and Zivkovic 2020).

In this paper the optimization of two important parameter of ELISA are discussed which can provide insight for shortcoming the problems of blocking and matrix effect while performing the ELISA.

MATERIAL AND METHODS

Sample collection and processing: Blood samples from buffaloes and cows were collected after artificial insemination (cattle yard, NDRI). Blood samples were collected through jugular vein into 10 mL evacuated serum collection tubes (Vacutainer; BTL Research lab Vadodara). Samples were centrifuged at 4ºC at 2500 rpm for 20-25 minutes. The obtained serum samples were kept at -80 ºC until used for ELISA. All ethical guidelines were followed during sample collection process.

Hardware's: Nunc MaxiSorp flat-bottom clear 96 well plate (Thermo # 44-2404-21) were used. Thermo scientific (30-300 µl) and eppendorf research (100 µl) multi-channel precision pipette with disposable plastic tips were used. NanoQuant infinite 200 PRO plate reader was used to detect the substrate based fluorescence signal. Thermo Scientific XIR refrigerated centrifuge for processing the blood samples to isolate serum. Dual refrigerator shaker (M/S MRC limited) was used for incubation of plates. –4°Crefrigerator was used for overnight incubation of coated plate.

Reagents: Coating buffer: 0.2 M sodium carbonate/bicarbonate, pH 9.4. Capture antibody: BuPAG-1 monoclonal antibody diluted in Coating Buffer. Wash buffer: PBST (Phosphate buffer saline with 0.05% Tween20) wash buffer was used for washing plate. Blocking buffer: Phosphate buffer saline + 0.05% Tween 20 (PBST), 1% Bovine serum albumin (BSA), 1% boiled Bovine serum albumin (BSA) in Wash Buffer. Samples/standards: Serum samples collected from cows and buffaloes were used for detection. Primary antibody: BuPAG-1 polyclonal antibody diluted in PBST Buffer. Secondary antibody: HRP-conjugated specific antibody (raised in rabbit) diluted in PBST buffer. Substrate: 3, 3', 5, 5'-Tetra-methyl-benzamine (TMB) liquid substrate purchased from sigma was used. Stop solution: 2M sulfuric acid was used.

Procedure of Developing ELISA. Capture antibody was diluted in 0.2 M sodium carbonate/bicarbonate buffer (mH 9.6) to the appropriate concentration (1µg and 5 µg) and also at different volume of 50µl, 100 µl, 200 µl per well. Diluted capture antibody was added to the plate, was covered with plate sealer and incubated for 1 hour at 37ºC with continuous shaking in incubator (alternatively overnight incubation at 4ºC also performed). Next the plate was taken out and incubated at room temperature for 30 minutes. Content of the plate was discarded and washed with 250 µl PBST

wash buffer per well. 3-4 washing was done with shaking and each washing was of 3 minutes. Then added 100 µL of blocking buffer Phosphate buffer saline + 0.05% Tween 20, 1% Bovine serum albumin, 1% boiled bovine serum albumin per well), and covered the plate followed by incubation of 1 hour at room temperature (alternatively overnight incubation at 4ºC was performed). Blocking buffer was then removed and the plate was washed with PBST wash buffer of (volume 250 µl per well. 3-4 washing was done with shaking and each washing was of 3 minutes. Samples and standards (concentration: 0-320 ng/ml) were prepared and both were added in the same volume (50 µL) per well. Meanwhile 100-200 µL of primary detection antibody of concentration 800ng/ml was added and incubated for 2 hour at 37ºC with continuous shaking in incubator plate content was removed and plate was washed with 250 µl per well PBST wash buffer. 3-4 washing was done with shaking and each washing was of 3 minutes. 100µl of diluted labelled secondary detection antibody of concentration 100ng/ml was added to the plate, covered and incubated for 1 hour at 37ºC with shaking in incubator. Again the plate content was removed and plate was washed with 250 µl per well PBST wash buffer. 3-4 washing was done with shaking and each washing was of 3 minutes. Followed with that, 50 µl of TMB substrate was added and incubated for 10 minutes in incubator at 37ºC. After incubation the reaction was stopped by adding an equal amount (50 µl) of stop solution. The absorbance was measured at 450 nm using NanoQuant ELISA plate reader. Absorbance was measured at 450 nm using NanoQuant ELISA plate reader.

RESULTS

Optimization of blocking reagent: Blocking agents, such as bovine serum albumin (BSA), non-fat dry milk, and whole serum, are frequently employed to remove the empty binding space in the wells. Without proper blocking, the plate would bind both the antigen and the detecting antibody, producing a strong background signal and low sensitivity (Minic and Zivkovic 2020). Volume of blocking buffer used is usually double of the coating antibody volume to provide better blocking. In this study we have tried blocking with three different reagents: Phosphate buffer saline + 0.05% Tween 20 (PBST), 1% Bovine serum albumin (BSA), 1% boiled Bovine serum albumin (BSA). Since the plate coating was done for 100 µl per well, so for blocking volume of 150-200 µl per well was used.

Optimization of samples: In ELISA screening of samples is one of the crucial step. While using neat serum samples, matrix effect was observed. There are many components in serum samples, such as carbohydrates, proteins, and phospholipids that can interfere with the ability of the antibody pairs to bind to their target. This phenomenon is known as the "Matrix Effect". Due to these OD readings much lower than expected is obtained and all the samples were showing the similar readings (Table 1 (A)). To overcome it, the serum samples used in present study was diluted in 1:8 ratios in 1X PBST buffer (pH 7.4) and 50 µl of samples were used for detection.

Generating Standard curve: After optimizing the ELISA parameters, generation of standard curve having R^2 value close to 1 is required. As a result, standard curve was prepared for the target protein by plotting the mean absorbance (y axis) against the protein concentration (x axis) by making serial dilutions of the protein standard within a range of concentrations near the expected concentrations of the unknown samples. The ELISA standard curve was prepared by making serial dilutions of standard with known concentration in 1% boiled bovine serum albumin from the standard stock solution. Recombinant proteins of BuPAG1 (concentration = 0.6mg/ml) was used in order to develop standards. As per the standard protocol, seven dilution of standards was prepared and for constructing the regression fit curve the five best standards were used. To get R^2 close to 1, the standards were further optimized by bringing the concentration in ng/ml. Finally, the range of 0-320 ng/ml gave value of R^2 close to 1. The stock standard proteins of BuPAG1 was further serially diluted in 0.1% boiled BSA up to seven folds to obtain range having concentration from 0-320ng/ml. The equation $y = mx + b$; m = slope of the line and b = y intercept obtained from the curve was used to calculate the concentration of each sample by using the average of the duplicate samples for x in the equation.

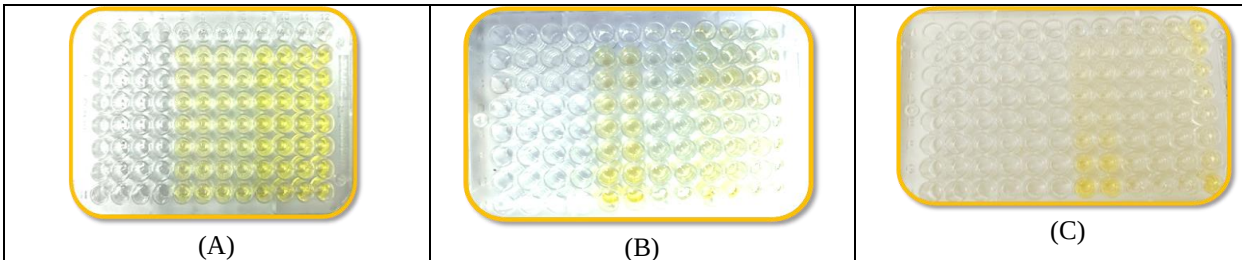

Fig. 1. Plate treated with different blocking reagents (A) Phosphate buffer saline + 0.05% Tween 20 (B) 1% Bovine serum albumin (C) 1% boiled bovine serum albumin.

Table 1: Matrix effect (A) Neat serum showing common readings due to matrix effect (B) Diluted serum showing appropriate reading.

SAMPLES O.D (matrix effect)	
0.07805	0.0814
0.0759	0.0853
0.07575	0.083
0.084	0.11045

(A)

SAMPLES O.D (without matrix effect)	
0.0289	0.16165
0.0239	0.1552
0.02565	0.16745
0.02265	0.1893

(B)

Table 2: O.D values of different samples used for generating standard curve.

CONC	OD1	OD2	MEAN	MEAN-BLANK
0	0.0481	0.0481	0.0481	0
5	0.1804	0.1881	0.18425	0.13615
10	0.1929	0.1917	0.1923	0.1442
20	0.2091	0.1908	0.19995	0.15185
40	0.2083	0.2188	0.21355	0.16545
80	0.2376	0.2523	0.24495	0.19685
160	0.2963	0.2867	0.2915	0.2434
320	0.3615	0.3776	0.36955	0.32145
ng/ml				

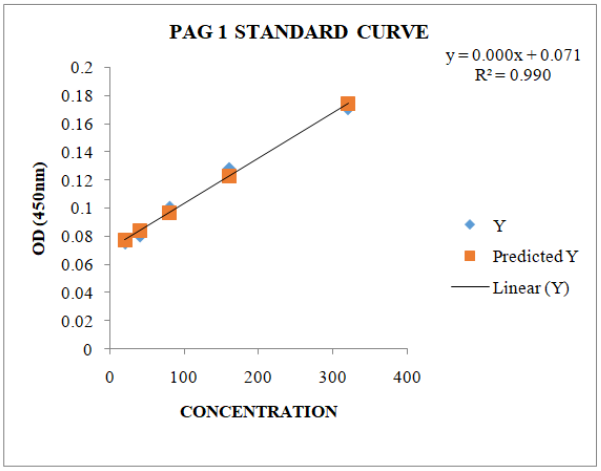

Fig. 2. Line plots by taking standard protein concentration on x-axis and OD on y-axis based on final optimization for ELISA assay. Obtained R^2 value was close to 1.

CONCLUSIONS

In order to avoid non-specific binding of antigens and antibodies to the microtiter well, bovine serum albumin (BSA) is utilized as a blocking agent (Xiao and Isaacs 2012). Additionally, blocking agents can minimize non-specific interactions and stabilize the biomolecules attached to the well surface (Gibbs and Kennebunk 2001).

Among the three blocking reagents, PBST showed higher background means excessive colour development. 1% BSA was used as blocking agent worked better than PBST. Since BSA is also from bovine origin (Majorek *et al.*, 2012), it may affect the binding as well as can show cross reactivity with serum samples of bovines. Thus, to overcome this we used boiled 1% BSA which showed the best result (Fig. 1) among the three blocking reagents used.

There are many components in serum samples, such as carbohydrates, proteins, and phospholipids that can interfere with the ability of the antibody pairs to bind to their target. This phenomenon is known as the "Matrix Effect". Due to this OD readings much lower than expected is obtained. Thus, dilution was best way to mitigate matrix effect and also allows the samples to fall under the range of standards. The selection of the diluent is crucial since samples must nearly always be diluted before being used in an ELISA test (Minic and Zivkovic 2020).

It can be clearly observed from the results that matrix effect can be overcome by diluting the samples in 1:8 ratios in 1X PBST buffer (pH 7.4) so that one can achieve proper optimization in terms of OD reading for detecting the target samples.

It can be clearly observed from the results (Table 1 (B), that matrix effect can be overcome by diluting the samples in proper ratio so that one can achieve proper optimization in terms of OD reading for detecting the target samples.

After optimization of ELISA, the standard curve generated has shown $R^2 = 0.9908$ (Fig. 2) which would be considered perfect. Thus, it was concluded that the above optimization along with optimization of antibodies concentration has worked best for generation of standard curve based on the OD of samples.

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