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DEVELOPMENT AND EVALUATION OF A RABBIT POLYCLONAL ANTIBODIES-BASED ELISA FOR THE DETECTION OF BOVINE PREGNANCY-ASSOCIATED GLYCOPROTEIN (PAG) AS A DIAGNOSTIC TOOL FOR BOVINE PREGNANCY

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ABSTRACT

The aim of this study was to develop a polyclonal antibody (Rabbit anti-BovPAG) capable of detecting PAG in the serum of pregnant cows. Five rabbits were immunized against synthetic peptide bovine PAG (BovPAG) preparations. Each 0.5 ml of PAG protein (1000µg) was reconstituted in 500µl Phosphate Buffer Saline (PBS) and emulsified in equal volume with complete and incomplete Freund's adjuvant.The mixture of immunizer and adjuvant was injected subcutaneously at multiple sites along the dorsal area of the rabbits. Blood samples were collected from the marginal ear vein, starting before the first injection (Baseline) and every 14 days after that. Rabbit anti-BovPAG levels were measured using indirect ELISA. It can be concluded that synthetic peptide bovine PAG (BovPAG) is a suitable source for producing specific rabbit anti-BovPAG antibodies and has the potential for development as a key antigen for antibody production as a base for pregnancy diagnosis in bovine kits.

Keywords:

ELISA, BovPAG, Polyclonal Antibody, Rabbitanti-BovPAG.

INTRODUCTION

Pregnancy-associated glycoprotein (PAG) is a member of the aspartic proteinasefamily. It is secreted by the trophoblast, a specialized cell layer derived from the fertilized egg **(Wooding, 1992; Perényi *et al.*, 2002; Hughes *et al.*, 2003).** PAG is expressed in the outer epithelial cell layer of the ungulate placenta, known as the chorion or trophectoderm **(Green *et al.*, 2000).**Immunological assays are a valuable tool for monitoring breeding programs in cattle. These assays rely on the detection of specific antigens secreted by specialized cells within the bovine uterus. When the blastocyst implants in the endometrium, these proteins enter the

bloodstream, serving as indicators of pregnancy **(Ball and Peters, 2004; Bearden *et al.*, 2004).**In the present study, pregnancy-associated glycoprotein (PAG) syntheticpeptide, has been demonstrated to be immunogenic **(Zoli *et al.*, 1991).** Immunogenic proteins, when introduced into laboratory animals, stimulate the immune system to produce specific antibodies **(Abbas *et al.*, 2000).** Our objective is to develop an antibody against PAG for its application in immunological assays for pregnancy detection in cattle.

MATERIAL ANDMETHODS

Rabbit Immunization and Preparation of Polyclonal Antibodies against Bovine Pregnancy-Associated Glycoprotein (BovPAG).

The immunization dose of BovPAG used as an antigen was 500 µl, containing 1000 µg per rabbit. This dosage aligns with the procedure established by **Hudson and Hay (1989),** where the typical hormone immunization dose in rabbits ranges from 50 to 1000 µg/ml **(Hanly *et al.* 1995).** All the experimental procedures were carried out following good veterinary practices and approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Cairo University.

Peptide Synthesis and Conjugation:

Peptides were synthesized based on the amino acid sequences of Pregnancy-Associated Glycoprotein1-likeproteins.Onespecific peptide wasidentifiedas bovine Pregnancy-Associated Glycoprotein (BovPAG).

Conjugation for Different Applications:

- **Animal Immunization:** The BovPAG peptide was conjugated to ovalbumin (OVA) to serve as an immunogenic for animal immunization. This conjugate stimulates the immune system of the animal to produce antibodies specific to BovPAG.
- **ELISA Coating Antigen:** A separate batch of the BovPAG peptide was conjugated to bovine serum albumin (BSA) to create a coating antigen for Enzyme-Linked Immunosorbent Assay (ELISA). This conjugate allows the specific capture of anti-BovPAG antibodies during the ELISA test.

Immunization Procedure:

Five male rabbits, approximately 2 months old, were used for the production of anti-BovPAG antibodies.

Primary Immunization (Day 0): Each rabbit received a subcutaneous injection of 1000 µg BovPAG, reconstituted in 500 µl PBS and emulsified in an equal volume of complete Freund's adjuvant (CFA) (Sigma). The injection was administered at multiple subcutaneous (S/C) sites **(Singh *et al.*, 2003).**

Control Group: One rabbit received an injection of 500 µl Phosphate Buffer Saline (PBS) diluted in 500 µl CFA as a control.

Blood Collection:

Before immunization, a baseline blood sample (Negative Control) was collected from each rabbit.

Booster Injections:

The first booster injection was administered two weeks after the primary immunization using Freund's Incomplete Adjuvant (Sigma). Subsequent boosters followed the same protocol at two-week intervals.

Blood Collection and Serum Storage:

After the sixth booster injection, blood was collected from each rabbit. The serum was separated, aliquoted into small portions, and stored at -20°C until further use.

Measurement of Rabbitanti-BovPAG Concentration using ELISA Technique:

BovPAGprotein (50, 100, 200, 400, and 800 µg/ml) was diluted in carbonate-bicarbonate buffer (Coating Buffer) at a 1:10 ratio.

100 µl of each diluted BovPAG sample was added to each well of an ELISA plate.

The plate was incubated at 4°C overnight. The plate was washed four times with PBS containing 0.1% Tween 20 (PBS-T).

300 µl of 5% Blot solution (5% non-fat dry milk in PBS-T) was added to each well. The plate was incubated at 37°C for 60 minutes. The plate was washed four times with PBS-T. Rabbit

anti-BovPAG antibody was diluted 1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600 in 5% Blot solution.

100 µl of the diluted antibody solution was added to each well.

The plate was incubated at room temperature for 90 minutes. The plate was washed four times with PBS-T.

Goat anti-rabbit IgG peroxidase conjugate (KPL®) was diluted 1:1000 in a 5% Blot 100 µl of the diluted conjugate solution was added to each well.

The plate was incubated at 37°C for 60 minutes. The plate was washed four times with PBS-T.

100 µl of TMB substrate (3, 3', 5, 5'-tetramethylbenzidine dihydrochloride) (Sigma®) was added to each well.

The plate was incubated at room temperature for 20 minutes, allowing the color to develop blue.

The reaction was stopped by adding 50 µl of 2N sulfuric acid (H2SO4) to each well. The color changed from blue to yellow, indicating the end of the reaction.

The optical density of the yellow solution was measured at 450 nm using a Microplate Reader Model 3550 (Bio-Rad®) **(Crowther, 2001; Cho *et al*., 2003).**

Data Analysis:

Immune responseofrabbit anti-Bov PAG were analyzed descriptively. The Response was analyzed by measuring the changing of optical density BovPAG to rabbitanti- Bov PAG (50, 100, 200, 400, 800, and 1600) gained during immunization. Based on responseimmune and total protein concentration, Rabbitanti-Bov PAG was chosen as ameasuring Bov PAG concentration in serum.

RESULTS ANDDISCUSSION

An immunogen, a substance that triggers an immune response, follows specific criteria. It must be a foreign agent (From A different Species), have a large molecular mass (Complex structure), and be administered through a specific dose, injection route, and timeframe. Additionally, the immunogen should have epitopes (Antigen Sites) for antibodies to bind and be capable of inducing a specific immune response. Rabbits are frequently chosen for antibody production due to their different species status, ease of handling, and lack of prior exposure to the target antigen. In this case, Bovine Pregnancy-Associated Glycoprotein (BovPAG) fulfills

these criteria and has never been injected into the rabbits chosen for antibody production **(Klisch *et al.*, 2005).**Knowing the specific epitopes (Active Binding Sites) of an antigen (BovPAG) allows detection of B or T lymphocytes that can attach to them. The immune response generated during immunization depends on the injected dose and route. If multiple epitopes are present, a mixture of antibodies (Polyclonal Antibodies) can be produced, as described by **Goldsby *et al.* (2000**) **and Abbas *et al*. (2007).** The strength of the interaction between antigen and antibody depends on their affinity. Higher affinity translates to a stronger binding ability, allowing the antibody to bind the antigen for a longer duration. Cross-reactivity occurs when two antigens share identical epitopes, or when an antibody's specific binding site for one epitope also binds to another epitope with a similar chemical structure **(Huebner, 2004).**

Standardization of the ELISA method:

A checkerboard titration was conducted to optimize the indirect ELISA reaction **(Crowther, 2001).** The antigen were coated to plate with different concentrations; 50, 100, 200, 400, and 800 µg/ml. Serum concentrations were diluted with blocking buffer at different dilutions;1:50, 1:100, 1:200, 1:400, 1:800, and 1:1600. The HRP Goat-anti-rabbit IgG were also diluted with blocking buffer at different dilutions; 1:1000. The checkerboard titration method was conducted using positive and negative samples as described above (Table 1).

Table (1): The checkerboard titration to determine the optimal reaction of an in-house ELISA.

Ag conc. (µg/ml)	Serum dilutions with blocking buffer					
	1:50	1:100	1:200	1:400	1:800	1:1600
50.	5.879	6.717*	3.186	2.536	1.584	1.414
100.	5.663	4.894	4.294	4.219	3.688	2.196
200.	4.562	4.035	3.854	4.041	3.612	2.534
400.	4.128	3.552	3.372	3.229	2.811	2.356
800.	3.100	3.405	2.120	3.010	1.240	1.100

The table shows the sample per negative (S/N) ratio. The conjugate used onedilution,1:1000.* Represents the suitable S/N ratio from this study.

Measuring *Rabbit anti-Bov PAG*C oncentration:

Due to variations in immune response among the five rabbits (n=5) during immunization, antibodies for use as polyclonal sources must be carefully selected based on their effectiveness. Five rabbits showed a good response to BovPAG as measured by optical density using an indirect ELISA. The baseline titer refers to the level before the first Bov PAG injection. To enhance Bov PAG's immunogenicity, the isolate was combined with an adjuvant. Complete Freund's adjuvant (Sigma®) containing *Mycobacterium* sp was used for the first immunization only. For the second and third immunizations, rabbits received a dilution of BovPAG with incomplete adjuvant. These injections stimulated B and T cells to produce an immune response, resulting in rabbit anti-BovPAG antibodies **(Cruse and Lewis, 2002).**

The first immunization triggered B-cell proliferation and differentiation into antibody-secreting and memory cells, initiating the primary immune response. Some antibody-producing cells migrated to the bone marrow for long-term residence. The second (Booster I) and third (Booster II) immunizations led to a secondary immune response, with higher antibody concentrations compared to the first immunization. This occurred because memory B cells were activated by T cells, leading to a larger antibody production **(Goldsby *et al.*, 2000; Abbas *et al.*, 2007).**

Importantly, the control group, which received only PBS injections, showed a low optical density, while groups immunized with BovPAG produced an immune response. This indicates that rabbits recognized BovPAG as a foreign agent (Antigen).

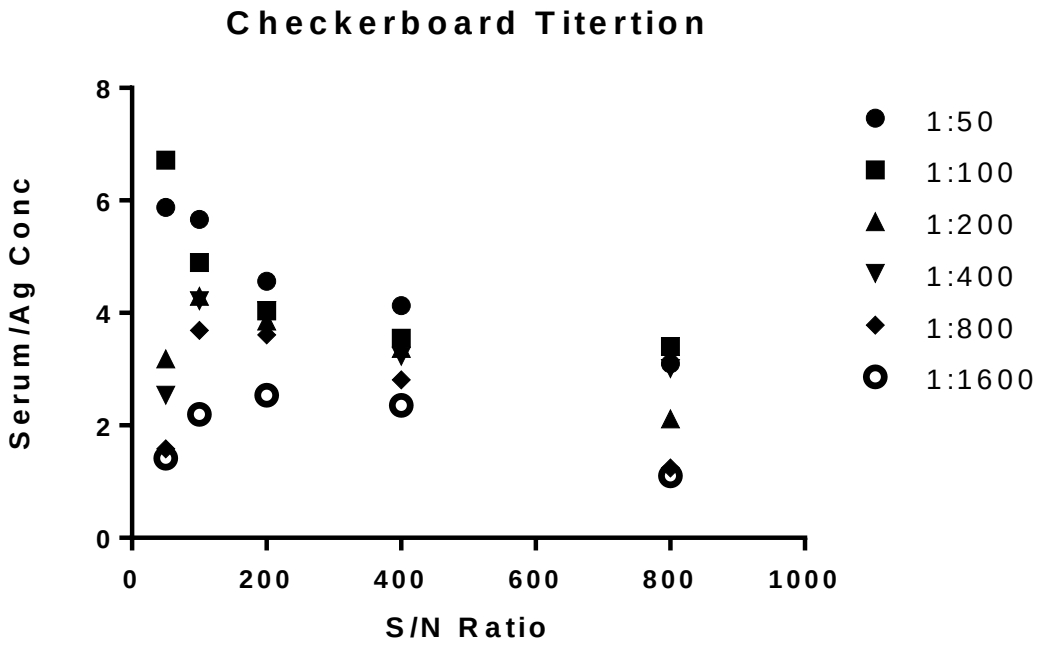

Fig. (1): Depicts a suitable signal-to-noise ratio obtained in this study.

CONCLUSION

This study demonstrates the successful production of polyclonal antibodies from synthetic peptide Bov PAG. To develop more applicable pregnancy detection tools, further research is warranted to explore the use of these antibodies in ELISA technique.

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