

## STUDY OF THE ANTI – PREGNANCY - ASSOCIATED GLYCOPROTEIN (ANTI – PAG) RESULTED FROM ANTIGEN PAG IMMUNIZATION, AS PROSPECTIVE EARLY PREGNANCY DETECTOR IN ANIMALS

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**ABSTRACT.** Blastocyst protein named pregnancy-associated glycoprotein (PAG) has been isolated from pregnant dairy cow serum. PAG yielded from previous research issued from pregnant animals, wherein was specifically produced. This study is an initial research to use PAG as an antigen to be injected to rabbit in order to produce the corresponding antibody. The objective of the research was to study the character of anti-pregnancy-associated glycoprotein (Anti-PAG), as a result of immunization of PAG and to learn its specification reaction. The PAG isolate had bovine origin (molecular weight-MW of 67.34 kDa) and issued from previous research. Injection of PAG isolate could stimulate the production of Anti-PAG as an immunization response. Immunization was done by double booster inoculation. Anti-PAG derived from immunization was characterized via antibody titer value using ELISA technique. Specificity test of Anti-PAG was carried on by Western Blot technique. Results revealed

that injection of PAG isolate to the rabbit stimulates anti-PAG production. Concentration of Anti-PAG was 1.192 µg with titer of 1.044. Anti-PAG derived from rabbits recognized Standard PAG antigen (bovine PAG MDBiomed cat. 101-7963-13-3) and PAG isolate derived from previous research. The reaction of antigen antibody is the basic structure of creating a gestation detection kit. Anti-PAG is expected to be the molecular marker in developing a gestation detection kit in the near future.

**Keywords:** protein pregnancy-associated glycoprotein (PAG); cow serum; immunization; rabbit; Anti-PAG.

### INTRODUCTION

Detection of early pregnancy is one of the main keys to achieve successful breeding programme and is a basic control method of animal

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infertility. Development of alternate, cheap, easy to run and accurate methods for early pregnancy detection in heifers is still in progress. Existing clinical detection methods comprise rectal exploration or obstetric ultrasonography, which are effective only if 40 days of gestation have passed. However, it is necessary to have a method for early pregnancy detection, especially for heifers. In the dairy farming, 25% replacement of the dairy herd must be replaced yearly by heifers. A heifer will cost higher if pregnant. To sell a pregnant heifer will be beneficial for both seller and buyer, because it means the heifer is fertile and the buyer will obtain the calf as well. Therefore, it is necessary to identify an appropriate method of earlier pregnancy detection. One of the ways to detect pregnancy earlier is to use immunogenic assays and all the efforts must focus on to that direction.

Immunogenic detection of early pregnancy involves existing specific proteins produced by blastocyst on the early implantation stage. As soon as the blastocyst implants to endometrium, its specific proteins pass into the maternal bloodstream (Jainudeen and Hafez, 2000). Specific protein detected in the cow blood serum after mating of artificial insemination could be used as a gestational indicator (Ball and Peters, 2004; Bearden *et al.*, 2004; Hafez, 2000).

Some proteins produced by the blastocyst are: bovine Pregnancy Specific Protein B (bPSPB), bovine Trophoblastic Protein-1 (bTP-1),

Pregnancy Specific Protein 60 (PSP60), Early Protein Factor (EPF) and Pregnancy-Associated Glycoprotein (PAG) (Gordon, 2004). Pregnancy-Associated Glycoprotein (PAG) contains glycoprotein molecules produced by trophoblast cells of blastocyst when implantation occurs after conception (Klisch, 2005; Majewska *et al.*, 2009; Xie *et al.*, 1991).

PAG was believed to have immunogenic characteristic (Klisch *et al.*, 2005; Xie *et al.*, 1991; Zoli *et al.*, 1990a,b). All immunogenic proteins, when injected into the laboratory animals will stimulate the production of the corresponding specific antibody (Abbas *et al.*, 2000; Gordon, 2004). As a specific antigen, PAG is a glycoprotein and has acid characteristic (pH 4.4 - 5.4) with molecule weight (MW) of 67 kDa. Immunogenic substance, which has the molecular weight (MW) more than 10 kDa can stimulate the antibody production when the laboratory animal is specifically immunized (Abbas *et al.*, 2000; Abbas *et al.*, 2005; Goldsby *et al.*, 2000). This research had as main goal to produce the antibody PAG and to test it with specific reactions to prove whether the anti-PAG can recognize or not the antigen PAG. Anti-PAG is expected to be able to be used as a molecular marker of early pregnancy detection in dairy cows. In terms of producing large scale kits for laboratories, further research is needed.

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### MATERIALS AND METHODS

This research used PAG isolated from pregnant dairy cow serum (from previous research), which contains protein 4740 µg/ml and it was used as an antigen to immunize the rabbits. The dose of PAG as an antigen for immunization is 200 µl, and it is equivalent with 948 µg for each rabbit. This dose is in accordance with the protocol used by Aulani'am (2005). It is said that the dose of antigen immunization in the rabbit is between 50-1000 µg.

The production of anti-PAG antibody was organized on five male rabbits weighing 3 kg each and aged 6 months. They were firstly immunized on the day-0, inoculating subcutaneously 200 µg of PAG isolate, diluted in 200 µl complete Freud's Adjuvant (CFA) solution. One rabbit injected with 200 µl of PBS (Phosphate Buffer Saline) diluted in 200 µl CFA was used as control.

A day prior to first immunization (day-0) 5 ml of blood were sampled from *auricularis* vein of each rabbit. Fourteen days later, first booster was applied through inoculation of 200 µg PAG protein diluted in the 200 µl Incomplete Freud's Adjuvant (IFA) solution to the five treatment rabbits. The control rabbit was inoculated with 0.2 ml PBS in the IFA solution. Second booster was given to the five treatment rabbits on the 49<sup>th</sup> experimental day Aulani'am (2005).

Purification of anti-PAG was done by adding SAS 50% into rabbit serum with ratio 1:1, homogenized on a lab vortex. Further specificity test was carried on by Western Blot technique. Gel derived from SDS-PAGE was transferred to NC membrane in 90 voltage, overnight in a cold room. Transferred band in NC membrane was colored with ponceau stain, then blocked with 5% BSA in PBS

for one hour. Then, the NC membrane incubated with secondary antibody.

Concentration of Anti-PAG was measured by converting the data of absorbance using standard antibody and calculation using the simple regression equation below:

$$Y = b_0 + b_1X,$$

where X = anti-PAG content (µg/ml) and Y = absorbance value.

Titer of anti-PAG was characterized by ELISA indirect.

### RESULTS

The result of anti-PAG titer is shown in *Fig. 1*. It proves that the pre-immune rabbit (Pi) or rabbit without PAG immunization had absorbance value very low, compared with the one immunized by PAG, which reached 0.079.

It means that the blood serum of the rabbit before immunized by PAG (control) has no anti-PAG contained, as shown also in Pi coulomb in *Fig. 3*, where no band is detectable. Anti-PAG titer highly increased after booster II. The highest anti-PAG titer in booster I has the best absorbance value, i.e. 1.044 contains 1.192µ/ml, as mentioned in *Table 1*. The calculation derived from curve mentioned in *Fig. 2*.

Specificity test using Western Blot technique has been conducted to accomplish the research. This technique was used to prove that there is a reaction of antigen antibody between PAG antigen and anti-PAG, therefore to find the ability of PAG and anti-PAG mutual recognition. It was proved that anti-PAG derived

from immunization was recognized by the standard. The result of Western Blot test is revealed in Fig. 3.

Fig. 3 shows that anti-PAG produced from this research could be recognized either by the PAG derived from former isolation and by the standard PAG (bovine PAG MD

Biomed cat. 101-7963-13-3), as showed in band of rows 1, 2 and 3. Based on the result showed at Western Blot bands, it could be stated that there is an immunologic reaction between PAG and anti-PAG, therefore PAG and anti- PAG can recognize each other.

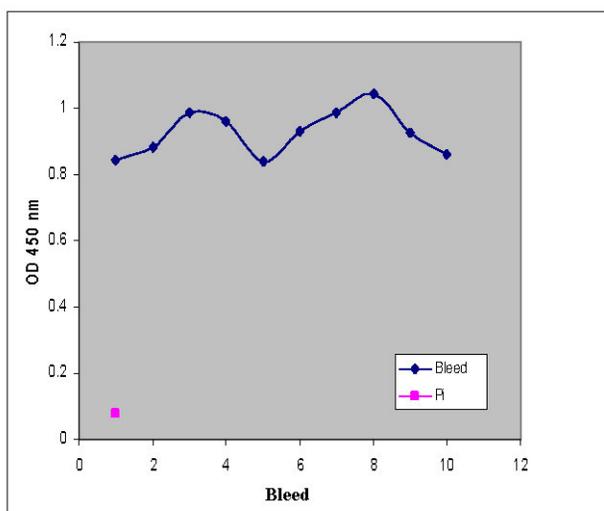


Figure 1 - Anti-PAG titer of isolation result from the rabbit

Table 1 - The absorbance value of Anti-PAG

Blood collection	OD 450 nm	X Concentration of Anti-PAG ( $\mu\text{g/ml}$ )
Pi	0.079	0.076
B1	0.843	0.960
B2	0.881	1.004
B3	0.988	1.128
B4	0.963	1.099
B5	0.841	0.958
B6	0.93	1.061
B7	0.986	1.125
<b>B8</b>	<b>1.044</b>	<b>1.192</b>
B9	0.924	1.054
B10	0.861	0.981

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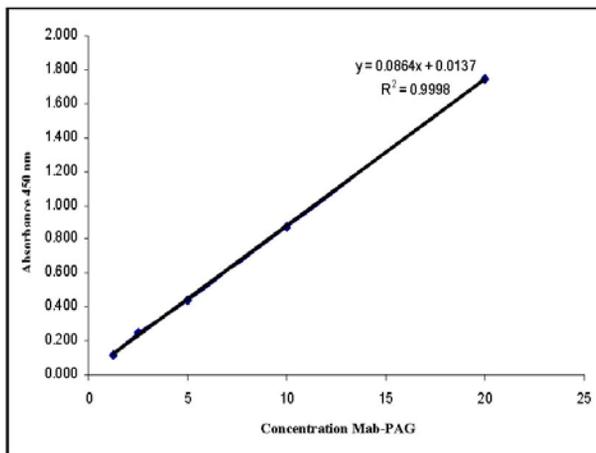


Figure 2 - The curve of standard anti-PAG

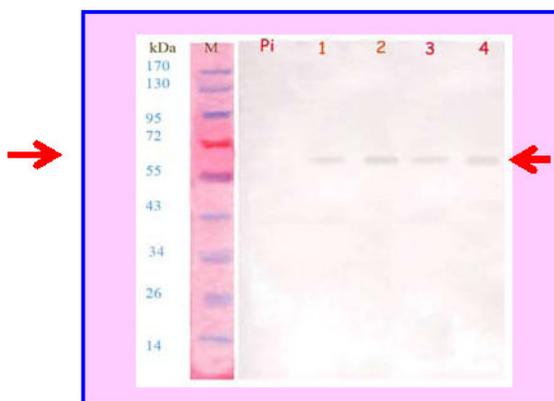


Figure 3 - Specificity test of PAG versus anti-PAG, via Western Blot Technique

PAG identified by anti-PAG / Mab – PAG; Pi: Serum of pre-immune rabbit  
1: band of PAG recognized by anti-PAG derived from immunization; 2: band of PAG recognized by Mab - PAG or standard anti-PAG (Anti-bovine PAG US Biological Cat. P2008-02); 3: band of PAG Standard (bovine PAG MDBiomed cat. 101-7963-13-3) recognized by anti-PAG derived from immunization; 4: band of PAG Standard (bovine PAG MDBiomed cat. 101-7963-13-3) recognized by Mab – PAG (Anti-bovine PAG US Biological Cat. P2008- 02)

## DISCUSSION

Antibody is a protein molecule produced by plasma cell as a consequence of interaction between B lymphocyte and specific antigens (Abbas *et al.*, 2000; Tizard, 2004). The antibody has an ability to bind a specific antigen. Antibody exists in several body liquids but the highest concentration is in blood serum, from where is the easiest to collect (Tizard, 2004). The use of protein in term of antibody production yields polyclonal and monoclonal antibody with different specifications, depend on epitope expression on the protein surface (Tizard, 2004).

Antigen is a substance which can be recognized and bounded by immune system (Rantam, 2003). Antigen could be derived from bacteria, virus, fungi and parasites or molecules, such as proteins, compounds, which are strange for the body. This research used pregnancy-associated glycoprotein (PAG) as an antigen. Immune response toward the antigen is happened firstly because the macrophage known as antigen-presenting cell (APC) turns the antigen into a peptide. This peptide binds protein major histocompatibility complex (MHC) class II and is presented on the top cell surface (Tizard, 2004).

PAG isolate from former research has MW of 67.34 kDa (Lestari, 2009). PAG is an immunogenic protein (Xie *et al.*, 1991; Zoli *et al.*, 1990a,b) and all immunogenic protein, which has

molecular weight above 1000 Daltons, when injected to animal laboratory, will stimulate antibody production towards the protein itself (Abbas *et al.*, 2000; Goldsby *et al.*, 2000).

Antibody production involves the use of laboratory animals, such as rabbits, guinea pigs and mice (Aulanni'am, 2005). These laboratory animals can produce 25 ml, 100 - 200  $\mu$ l and 1-2 ml serum blood to be collected. This research used rabbits, in order to produce enough serum to be analyzed, easy to handle and cheap, in addition, quite safe to sample several times through bleeding.

The production of anti-PAG antibodies in the rabbits could be explained basing on the theory that the antibody is the protein molecule produced by plasma cells because there is an interaction between the lymphocyte B antigen-sensitive and the presence of the antigens (Abbas *et al.*, 2000; Tizard, 2004). The processes could be resumed in brief as follows: PAG protein entered into the body of laboratory animals and was processed in the Antigen Presenting Cells (APCs), then presented to the receptors of the T cells, connected by major histocompatibility complex (MHC) class I and II. APCs produce and release cytokinine, stimulating the T cells to proliferate and differentiate. Then, following the chain immune reaction, the T cells were stimulated to release their own cytokinine, which activated B cells on three levels: activation, proliferation, and

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differentiation into plasma cells, which produce Ig (immunoglobulin). The T cells receptors is very specific to the configuration of certain peptides / MHC and every T cells only has a receptor type to make sure the immune response is strictly specific. B cells have bound the PAG protein and bound together. Plasma cells then produce anti-PAG. According to Tizard (2004), antibody titer post booster II is higher than post booster I, because the body has memory to identify antigen which has entered before (Xie *et al.*, 1991).

Based on specificity test, anti-PAG has been recognized by antigen PAG standard, as well as antigen PAG derived from former research. It means anti-PAG could be used as a kit to detect PAG in circulating blood of early pregnant cow, as in this situation, the blastocyst produces protein, i.e. PAG.

### CONCLUSIONS

Protein produced by trophoblastic cell of blastocyst, named pregnancy-associated glycoprotein (PAG), is one of protein which exists in pregnant animals serum. PAG has MW of 64 kDa. Antigen, which has MW more than 10 kDa, is able to stimulate antibody when injected to animal laboratory.

One of the pregnancy detection technique is based on immunology, using the antigen - antibody reaction. Antibody Anti-PAG, derived from immunization, is expected to be a molecular marker in detection of

pregnancy, hence in a serum of pregnant cow there is an antigen of PAG.

Injection of PAG Antigen to rabbits has triggered antibody production response (anti-PAG).

Antibody (anti-PAG) has recognized both antigen PAG standard and antigen PAG isolated from previous research.

The highest anti-PAG titer was harvested from the 8<sup>th</sup> collected blood sample in booster II, i.e. 1.044 and it contained 1.192 µg/ml.

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