Isolation and Characterization of Antibody Induced by 71 kDa Bovine Protein (Anti-P71) from Pregnant Cow Serum for Detection of Pregnancy in Cattle

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***Abstract*- The efficiency of animal production is important from a physical and economic point of view for livestock breeders, and reproduction is considered an important matter. Therefore, detecting the occurrence of pregnancy early after mating in cattle is very important to ensure the success of pregnancy and the preservation of the fetus until the birth of the new offspring, and confirming the occurrence of pregnancy as soon as possible is very important to preserve the sperm. Remarriage after pregnancy leads to the death of the fetus. Therefore, the main objective of this study was to isolate Pregnancy Associated Glycoproteins and use it to detect pregnancy in cattle in the shortest possible time after mating.**

The bp71 kDa protein was isolated using "SDS-PAGE" where the proteins are separated depending on the difference in molecular weight, and then the Pregnancy Associated Glycoproteins was extracted from the gel using the electroelution technique. After extracting the Pregnancy Associated Glycoproteins mixed with adjuvants and then injected into experimental animals to stimulate the immune system of those rabbits, which in turn produce antibodies to the Pregnancy Associated Glycoproteins of cows, and fore confirm the formation of antibodies, the indirect ELISA technique was used to determine the amount Antibodies formed and the time period required for the highest production of antibodies. Also, Western blot technique was used to ensure the ability of the antibodies to identify the Pregnancy Associated Glycoproteins bp71 kDa. the antigen used was PAG71. The primary antibody used was PAG71 antibody induced by PAG71 derived from rabbit serum, while the secondary antibody used was anti-Rabbit AP Conjugated.

the results of serum protein bands in pregnant cows, protein 71 kDa was found in the serum of pregnant cows aged 2 months, 5 months, 6 months, and 7 months, and in the serum of non-pregnant cows there was no protein 71.the result of western blot protein bands with a molecular weight of 71 kDa were seen in a sample of 7-month-old pregnant cows, while in non-pregnant cattle samples, no protein was found on protein bars of 71 kDa molecular weight. Protein bands with a molecular weight of 71 kDa were seen in a sample of 7-month-old pregnant cows, whereas in non-pregnant cattle samples, no protein bands of this molecular weight were found. In Conclusion, SDS-PAGE confirms the association of the protein PAG with cattle pregnancy, as mentioned in previous studies, and the Pregnancy Associated Glycoproteins appears in the range of 50 to 100 kDa. in western blot technique, correlation was observed Antibodies to pregnancy-associated protein in the serum of pregnant cows did not appear in the serum of non-pregnant cows.

***Index Terms*- indirect ELISA, Cows Pregnancy, PAG71, Western Blot, PSP-B.**

INTRODUCTION

Modern farming depends heavily on the ability to recognize pregnancy in cattle since it allows producers to manage their herds more effectively and assure healthy calving. Yet, the approaches used today are frequently ineffective or intrusive. Anti-P71, an antibody generated by P71 kDa bovine protein present in pregnant cow blood, has fortunately been identified as a promising new biomarker by current research. We may have discovered a more reliable and non-invasive approach for detecting pregnancy in cattle by isolating and identifying Anti-P71(1). One of the important factors for the success of the reproduction process in cows is the early diagnosis of pregnancy. pregnancy is diagnosed by one of two methods, either rectal palpation or ultrasound imaging (USG). These two methods have weaknesses, as ultrasound cannot be performed until a month after fertilization (2). and the rectal palpation method does not give a result except in pregnancy periods between 45 to 60 days of fertilization (3). There is continuing development of pregnancy diagnosis methods, the most important of which is an immunologically based method for the detection of pregnancy-related proteins, interferon, and progesterone, as well as Pregnancy-associated glycoprotein (PAG). This method is not accurate enough as there can be low specificity, and also false positives in some cows with fixed follicles where progesterone concentration increases despite no fertilization or pregnancy (4). the presence of PAG is also detected in cattle (cows, buffaloes, goats, and sheep) and some wild animals such as (deer and bison) during pregnancy (5). PAG belongs to the aspartic family protease, which is expressed by mononuclear trophoblast cells and bipolar trophoblast giant cells (BNCs) as an indicator of fertilization. PAG can be considered an indicator of fertilization, and the specific antigen for PAG is Pregnancy Specific Protein B (PSP-B) (6).

Detection of PSPB in bovine serum after mating can be relied upon as evidence or an indication that cows are pregnant during the period of zygote implantation (7).

MATERIAL AND METHOD

Serum samples were collected from a Green Farm Company at Pujon, Batu. From pregnant cows with different gestational ages of 2, 5, 6, and 7 months, and non-pregnant cows for the comparison group. 3 *New Zealand* White rabbits (6 months of age) from Bogor, were used for anti-P71 production. they were fed with dry food.

Sample Collection and PSPB Isolation

In this research, serum samples were collected from pregnant cows with different gestational ages of one month, two months, three, five, and seven months, and non-pregnant cows for the control and comparison groups. the cows should be in good physiological condition and body weight of about 350 kg (8). Blood serum was obtained from the jugular vein of pregnant PFH (Peranakan Friesien Holstein) cows aged 2, 5, 6, and 7 months which were reared by farmers by taking it using a 5 mL disposable syringe, then collected in a vacutainer tube with no additives. The tube is tilted 45° and left for 1-2 hours at room temperature. After that, the serum which had been separated from the blood was taken using a micropipette and transferred into a microtube and marked, then the serum will be separated again using a centrifuge at 6000 rpm speed for 30 minutes to get rid of all the insoluble substances in the blood. For isolation, PSPB using SDS-PAGE accordance with (9).

SDS-PAGE

Gel preparation is carried out by preparing a gel plate and two glass plates arranged with a distance between the plates of approximately 1 mm. Separating gel 14% for 1 gel was prepared by mixing 1350 µL of DDI H2O, 2350 µL of 30% Acrylamide, 1250 µL of Tris-HCL pH 8.8, 50 µL of 10% SDS and then waited for 10-30 minutes. Next, 50 µL of Ammonium Persulfate (APS) and 5 µL of TEMED were added and then inserted into the slot of the gel plate and then left for 15-30 minutes until the gel solidified. then prepare 7% Stacking gel for 1 gel made by mixing 2550 µL of DDI H2O, 1150 µL of 30% Acrylamide, 1250 µL of Tris-HCL pH 6.8, 50 µL of 10% SDS then wait for 15-30 minutes. Then 50 µL of Ammonium Persulfate (APS) and 5 µL of TEMED were added to the gel gap and then a gel comb was inserted to form a well where the sample was added and left to stand until the gel hardened. For sample preparation, take 5 µL of a serum sample, 100 µL of RSB, and 95 µL of Tris-HCL pH 6.8 with a ratio of 19:1:20 and then heat it in a water bath at 90°C for 5 minutes, wait until the sample cold and then attach the plate in the electrophoresis tool, the mini set of protein gel and the running buffer is poured into the tool to the limit. Then the samples were put into each well, with a volume of 20 µL for each well (9).

Antibody Production and Purification

Rabbit's immune system is stimulated by injection of antigen in the serum of pregnant cows, isolated protein (P71) with the addition of CFA and IFA as adjuvants will inject under the skin, 24 hours after the pre-immunization is given, the first immunization is performed and then the booster doses are given in the third week and fifth. An induction dose of 800 µl with 400 µl of adjuvant in a microtube containing 400 µl of antigen (bovine serum) p 71 and mixed using vortex for ±2 h until a completely homogeneous emulsion is formed (10). (Rahmatillah, 2015). Then the emulsion is injected in an amount of 1 ml under the skin, taking into account the difference in the injection sites between the first vaccination and the booster dose. Harvesting rabbit blood is done by drawing from a vein in the rabbit's ear with a 23-gauge needle. 3 ml of blood is withdrawn and then, the blood is collected in a vacutainer tube and placed at room temperature at a temperature of 45 degrees inclination until the serum is separated from the red blood cells, then the serum is withdrawn and placed in a centrifuge at a speed of 10,000 rpm for 15 minutes, then transferred to a micro-tube and kept at a temperature of -20 ° C.

week	Procedure	Adjuvant
1	Bleeding Pre-immune 0	**Pre-immune**
	Immunization	**CFA**
2	Bleeding pre-immune 1	
3	Bleeding pre-immune 2	
	Immunization 1	**IFA**
4	Bleeding 1	
5	Bleeding 2	
6	Bleeding 3	
7	Bleeding 4	
8	Bleeding 5	
	Immunization 2	**IFA**
9	Bleeding 6	
10	Bleeding 7	

11	Bleeding 8	
12	Bleeding 9	
13	Bleeding 10	

Table 4.1: Vaccination Schedule for Rabbits Injected with P 71- Pregnant Antigen Recombinant Protein Solution.

Detection of Antibody Titers by Indirect ELISA Immuno-dot Analysis

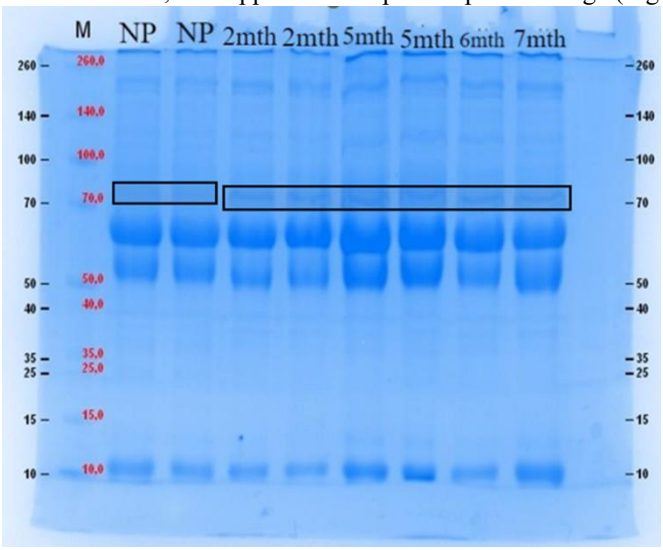
96-well *ELISA* plates are coated with recombinant P71 protein, dissolved in 1:9 coating buffer, placed in each microplate 100 µl, and the antigen is then incubated for 72 h at 4 °C. The wells are washed with 200 µl of washing buffer three times each time for 3 minutes. Using 100 µL/well of blocking buffer the antigen is blocked and incubated at room temperature for 2 hours. Then wash it again with washing buffer three times, each time for 3 minutes. A total of 100 µl of rabbit serum with a dilution of 1:100 is added to each well, incubated again at room temperature for 1 hour, and the wells are washed with washing buffer three times to remove unbound antibodies. To quantify the antibody in the plate, a secondary antibody is added, which is goat-anti rabbit IgG AP conjugate (1:2500), which has been previously incubated for 2 hours at room temperature. For imaging, pNPP 50 µL/well is added, covered, and left for about 30 minutes in the dark. The reaction was stopped by adding 3 M NaOH 50 µl/well. At a wavelength of 405 nm, the absorbance readings were performed on an ELISA reader (microplate reader).

P 71 Polyclonal Antibody Specificity Test Against P 71 Recombinant Protein with Western Blot (WB)

In the Western Blot assay, the first step is to transfer the antigen from a polyacrylamide gel using a system Trans-Blot® TurboTM to a nitrocellulose membrane. After the acrylamide gel is transferred to a nitrocellulose membrane using a semi-dry technique, the order is like a sandwich, that is, the bottom ion reservoir stack, the SDS-PAGE gel, and the top ion reservoir stack. Surface the membrane and gel in a cylinder with no air left and then soaked in blotting buffer pH 8.3 (28.8 g Tris base, 6.04 g glycine, and 400 mL methanol) at 200 V for 1 h. The nitrocellulose membrane was then rinsed with distilled water to confirm the successful transfer of the polyethyleneimine gel. Stained with CBB solution, using a buffer, the membrane is blocked for 1 hour, after which it is washed three times each time for 3 minutes with PBS (11).

RESULT AND DISCUSSION

Serum samples were collected from pregnant cows, which differ in gestational ages between 5,6,7, months, as well as non-pregnant cows for comparison, and then a profile of the serum protein profile was made, which showed the presence of a protein with a molecular weight of 71 kDa in the serum of pregnant cows, despite the difference Gestational ages. While in the serum of non-pregnant cows, it did not show a protein of 71 kDa, as it appears in the protein profile image (Figure 5.1), and despite that, protein



can be found in the range of 40 and 50 kDa, and also 100 to 200 kDa, in the serum of pregnant cows. While in the serum of non-pregnant cows, the protein appears between 50, 100, and 140 kDa.

Figure 5.1 Protein Band Profile of Serum of Cow PFH Bunting with SDS-PAGE method

The image shows the result of protein separation using electrophoresis and shows the presence of protein with a molecular weight of 71 kDa in pregnant cow samples at 2, 5, 6, and 7 months of pregnancy, while the absence of protein is noted in serum samples taken from non-pregnant cows.

Description: M= Marker, NP= Non pregnant, mth= Month

Based on the results of serum protein bands in pregnant cows, protein 71 kDa was found in the serum of pregnant cows aged 2 months, 5 months, 6 months, and 7 months, and in the serum of non-pregnant cows there was no protein 71. This is because Pregnancy Associated Glycoprotein 71 (PAG71) is secreted by cells-trophoblast cells so that they have the opportunity to detect early pregnancy because trophoblast cells begin to form in the blastocyst phase, starting on the 4th day of pregnancy, but there has not been contacting between the trophoblast and the mother so that PAG 71 cannot be found in the mother's blood circulation. By

the 7th day, the zona pellucida is around the hatching blastocysts, and on the 12th day, trophoblast cells have direct contact with the uterine epithelium so that PAG produced by placental trophoblast cells can already be found in the mother's blood circulation system (12). Pregnancy-associated glycoproteins 71 was only found in pregnant cows while in non-pregnant cows it was not found. In a study conducted by Klisch at 2005 protein weighing around 70 kDa in ruminants was shown to be produced by trophoblast cells and several large bands were detected, namely proteins of 75 kDa, 66 kDa, and 56 kDa(13). Research by Garbayo at 1998 showed the molecular weight of PAG in cattle, and goats at 67 kDa and 55 kDa at 48-69 days of gestation (14). Isolated ovine PAG (OV-PAG) with molecular weights ranging from 55-59 kDa below 50 days of gestation (5). Apart from Pregnancy-associated glycoproteins, several proteins are detected during pregnancy in cows, namely Placental Lactogen, a protein with a chemical composition similar to prolactin and growth hormone. The molecular weight ranges between 22,000 and 23,000 Daltons in sheep and consists of 192 amino acids. Placental lactogen can be found in humans, rats, goats, and cattle. This hormone is isolated from placental tissue in animals until the end of the gestational trimester (15). Next, Pregnancy Specific Protein B (PSPB) is produced by trophoblast binucleate cells from the bovine placenta. Pregnancy Specific Protein B (PSPB) can be taken from the tissue and fluid where the PSPB is located. Placental tissue is a good source because it has a high concentration of PSPB(11). Furthermore, bovine Trophoblastic Protein-1 (bTP-1), Pregnancy specific protein 60 (PSP60), and Early Pregnancy Factor (EPF).

Production of 71 kDa Antibody in Rabbits

The rabbits were injected with the purified protein by electrophoresis method, where the injection takes place in several stages starting with the first immunization by adding pregnancy-associated glycoproteins 71 (PAG71) to Complete Freud's Adjuvant (CFA) and Incomplete Freud's Adjuvant (IFA). The success of antibody production in rabbits was verified by ELISA, western blot, and dot blot. Rabbit serum was collected after immunization with (PAG71), as Pregnancy Associated Glycoproteins 71 in the body of rabbits were identified as a foreign body that does not remain in the body, and therefore the rabbit body interacts with it by increasing the immune response in the form of antibodies.

Sensitivity Test of Pregnancy Associated Glycoproteins 71 (PAG71) with Indirect ELISA Technique

The specificity selection procedure aimed to see if there was a response to Anti-P71 kDa that interacted directly and specifically with the P71 kDa antigen using its substrate as a reaction marker. Antibody (Anti-P71) requires testing of its specificity to determine the ability of antibody (Anti-P71) to recognize P71 antigen. Therefore, an introduction test was conducted for anti-P71 with P71 kDa, which we collected from the serum of pregnant PFH cows at the pregnant age of 7 months. The primary antibodies used were from rabbit blood serum before injection (stimulating immunity), and the results of rabbit blood collection from the first week to the 5th week after injecting with a dose of IAF 1 and from the first week to the 5th week after IFA 2. The result was the measurement of anti-B71 from the indirect ELISA as shown in Figure (5.2).

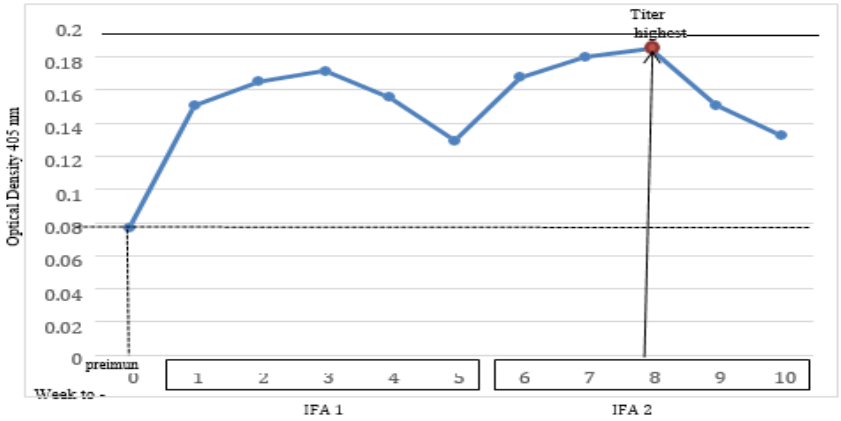


Figure 5.2. ELISA Absorbance Value

The curve shows the relationship between the concentration of antibodies produced by the immune system of rabbits in different periods of time, where the highest concentration appears in the eighth week after the booster dose in the fifth week.

Serum collected from rabbits before the immunosuppression dose induced the lowest anti-P71 titers. This is a natural result because the immune system has not been stimulated by an antigen of P71 kDa, and therefore the rabbit's body will not produce antibodies. The aim of measuring the highest titer using ELISA is to determine in which week the largest amount of antibody (P71) is produced, and according to the concentration of antibodies in the rabbit serum, the increase and decrease in antibody counters appear. A rise was observed after the dose of adjuvant IFA from the first to the third week, then it gradually decreased in weeks 4 and 5 (Fig. 5.2). Weeks 6 to 8. Antibody production was highest in week 8 after giving booster 2 doses in the 5th week, then decreased again in weeks 9 and 10. Immunization was performed again by reactivating the memory B cells to recognize the incoming antigen by producing specific antibodies. The secondary immunity created by the reboot can cause memory B cells to multiply faster, forming antibodies at a higher rate (16). and the results of the highest rate (week 8) will be used to detect pregnancy in cows.

Because the rabbits that were used in the experiment had a memory of the previous antigen, there was an increase in antibody titer after the booster immunization, so they showed specific antibodies. Ig G will appear in serum 6 to 7 days after exposure to previously recognized antigens (17). Innate immunity or the non-specific immune system is a naturally formed immune system that has no substantial effect from contact with previous infectious agents. This mechanism acts as a first line of defense, and at the same time, the specific immune system appears after stimulation due to a foreign body entering the body, causing the formation of a specific

immune system(18). The results showed that protein B71 has high specificity due to the presence of a link between protein B71 kDa and antibody B71, according to the results of OD on ELISA.

PAG71 Antibody Specificity Test Against PAG71 kDa by Western Blot Method

The specificity test of the PAG71 antibody in recognizing PAG71 derived from the serum of pregnant PFH cows was proven using the Western Blot method. In the Western Blot test, the antigen used was PAG71 protein isolated from the serum of 7-month-old pregnant PFH cows and the serum of non-pregnant PFH cows. The primary antibody used was PAG71 antibody induced by PAG71 derived from rabbit serum with the highest titer at bleeding week 8, while the secondary antibody used was anti-Rabbit AP Conjugated.

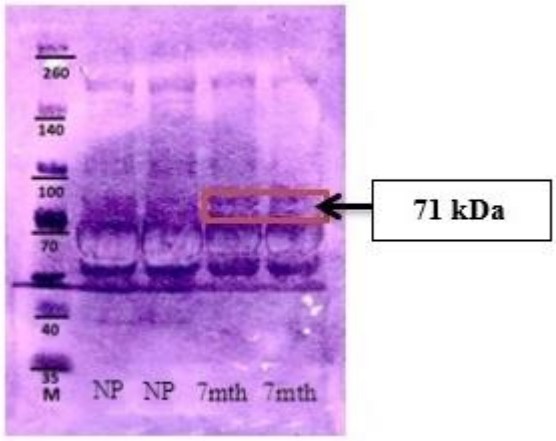


figure 5.3. Western Blot Antibody Bands PAG71 and PAG71

The picture shows the specificity test for the PAG71 antibody in the identification of PAG71 derived from the serum of pregnant PFH cows using the *Western Blot* method, the appearance of protein bands with a molecular weight of 71 kDa in a sample of pregnant cows aged 7 months, while in non-pregnant cattle samples, where no Protein bars with this molecular weight are not found.

Description: M= Marker, NP= Non pregnant, mth= Month

The results of the Western Blot test showed that the 71 kDa purple band was fragmented and could still be recognized on the nitrocellulose membrane. The molecular weight can be read using a protein marker reference with a molecular weight of 35 to 260 kDa. Protein bands at a molecular weight of 71 kDa were seen in a sample of 7-month-old pregnant cows, while in non-pregnant cattle samples, there is no protein bands were found at that molecular weight. Western blot results in non-pregnant cow samples did not reveal the presence of a protein in the 71 kDa range, and the reason for this is that there was no specific binding between the PAG71 and PAG71 antibody, as non-pregnant cows do not produce a protein PAG71. The results obtained from (SDS-Page) previously conducted that the protein band PAG71 induced in rabbit blood has a molecular weight of about 70.70 kDa, while the western blot test shows protein bands with the same molecular weight so it can be noted that the antibody PAG71 is specific because it can recognize PAG71 isolated from pregnant PFH bovine serum.

Western blot results in non-pregnant PFH cattle samples did not reveal any protein bands with a molecular weight range of 71 kDa, this was because, in non-pregnant cattle samples, there was no specific binding between PAG71 and PAG71 antibodies because PAG71 was not produced in non-pregnant cows. SDS-PAGE results that have been carried out previously showed that the PAG71 protein band induced in rabbits has a molecular weight of around 70.71 kDa, while the Western Blot test shows protein bands at the same molecular weight so that it can be seen that the PAG71 antibody is specific because it can recognize PAG71 isolated from pregnant PFH bovine serum.

CONCLUSION

In Conclusion, the separation results were confirmed by protein profile and protein levels with a molecular weight of 71 kDa for pregnancy in bovine serum at different gestational ages, while in the absence of serum from non-pregnant bovines. This confirms the association of the protein PAG with cattle pregnancy, as mentioned in previous studies, and the protein associated with pregnancy appears in the range of 50 to 100 kDa. Then the protein was extracted and injected with enhancers Complete Freud's Adjuvant (CFA) in experimental animals (rabbits) in order to stimulate their immune system to produce antibodies that will be used later to identify the protein in the blood of cattle to ensure the success of the mating process and the occurrence of pregnancy, and forecheck the concentration of antibodies we used the ELISA technique To find out the concentration of antibodies and the time period it took for the immune system in rabbits to form the antibody. After that, it was necessary to test the sensitivity of those antibodies to the protein extracted from the serum of pregnant cows using the western blot technique, as it gave good results and confirmed what was mentioned in previous studies, where the comparison was made in this test using the serum of non-pregnant cows and another pregnant one in the seventh month, and a correlation was observed Antibodies to pregnancy-associated protein in the serum of pregnant cows did not appear in the serum of non-pregnant cows.

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