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Characterization of Pregnancy-Associated Glycoprotein as a Biomarker of Pregnancy in Etawa Crossbred Goat

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ABSTRACT

Pregnancy-Associated Glycoprotein (PAG) is secreted by the placenta, which is produced in mononucleate and binucleate trophoblast cells. The current research was conducted to find out a substance for diagnosing early pregnancy in Etawa crossbred goats. Six Etawa crossbred goats (not pregnant, three months pregnant and four months pregnant) were subjected in the present study from Livestock Government Institution Breeding in Singosari, Malang. The research methods consisted of sample collection, identification PAG with sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the determination of concentration with Biuret method and specificity test with Western Blot assay. The obtained results showed that the molecular weight of PAG from pregnant Etawa crossbred goats was 55.85 kDa. The average concentrations of PAG in the goats of non-pregnant, three months pregnant, and four months pregnant were 1.83±2.98, 3.79±2.72 and 4.36±2.63, respectively. The results of the specificity test with the Western Blot molecular revealed a molecular mass of PAG was 55 kDa. The findings of the present study demonstrated PAG in Etawa crossbred goats can be used as an indicator of pregnancy.

Key words: Biomarkers, Etawa crossbred, PAG, Pregnancy

INTRODUCTION

Diagnosis of early pregnancy in goat can be done in two ways: through detection of specific substances in the peripheral circulation such as Pregnancy-Associated Glycoprotein (PAG) or non-specific substances in the blood, urine or milk such as progesterone and estrone sulfate (Hafez, 2000). PAGs are pregnancy indicators that are produced by mononucleate and binucleate trophoblastic cells (Perenyi et al., 2002; Karen et al., 2003; Sousa et al., 2006). Garbayo et al. (1998) purified three PAGs from goat placenta which differed in amino acid sequences and molecular weight (55 kDa, 59 kDa, and 62 kDa) and each of them had several isoforms with different isoelectric points. Isolation of ovine PAG was obtained at a molecular weight of 30.86 kDa from placental cotyledon (Setiatin et al., 2009). In cattle, PAG isolated from the blood serum during 274-279 days of gestation was characterized in molecular weight of 67.34 kDa (Lestari and Ismudiono, 2011). In livestock reproductive management, early pregnancy diagnosis is very economically advantageous in determining pregnancy status after mating (Restall et al., 1990; Goel and Agrawal, 1992). Generally, the length of the estrous cycle of the goat is around 21 days (Jainudeen et al., 2000). The economic losses of pregnant goats can be minimized or prevented by methods of early pregnancy diagnosis (Singh et al., 2004).

Pregnancy tests have the potential to be very suitable for field practice. PAG can be measured in maternal blood circulation (Shahin et al., 2013). Pregnancy is diagnosed using PAG test on day 24 of gestation (Reese et al., 2017). The knowledge of mechanisms involved in the production and control of PAG is beneficial in livestock breeding and facilitates diagnosis of pregnancy (Santos et al., 2018). Therefore, the current study was designed to evaluate blood serums for early pregnancy diagnosis in Etawa crossbred goats managed in intensive conditions in Indonesia.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Institutional Animal Ethics Committee Brawijaya University (Code No.1108-KEP-UB).

Collection of Samples

The present study was conducted on six crossbred goat aged 3 to 4 years in Livestock Breeding Institution Government in Singosari, Malang, East Java, Indonesia. The analysis of blood samples was conducted at the Department of Veterinary Reproduction Airlangga University, Surabaya and Biosains Laboratory, Brawijaya University, Malang,

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East Java, Indonesia. The goats were maintained under intensive system of management in well-ventilated pens and dietary and management conditions were the same for animals. Blood samples were taken from jugular veins of non-pregnant and pregnant goats in the different gestational age (3-4 month). The serum samples were centrifuged at 3000 rpm for 10 minutes and the supernatant was transferred in a new tube and was stored at -20°C until further use.

Identification of PAGs with Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The running gel was inserted into the SDS-PAGE tool through the wall to below the top line. Then, 1 ml butanol was added and left for 25 minutes. After the gel freeze, butanol was removed and cleaned with PBS then dried with Whatman paper. The 12% stacking gel is inserted from the top of the wall until it was fully set for 25 minutes. The comb was inserted and the remaining gel was cleared with a buffer. The samples were mixed with 5 µl of liquid buffer and heated at 100°C for 5 minutes. Then samples were inserted into the mold hole with a tip of 200 µl. The mold is inserted into an electrophoresis gel device, the power supply at the start was 125 V, 40 mA for one hour. When the electrophoresis was completed, it was turned off and the plate was opened and separated, then the gel was stained with Coomassie Brilliant Blue (Merck, Germany). Molecular weight determined using standardized regression between relative migration and molecular weight markers. Then, it was tested for specificity with Western Blots test.

Examination of PAGs levels using the Biuret method

The not-colored SDS-PAGE gel was cut to the desired tape. Each gel was inserted into a nylon sack and packed in a glass block containing PBS while were mixed on a magnetic stirrer for 24 hours. PBS was replaced every 6 hours. The gel pieces were stained with silver staining to detect protein. The total protein concentration was determined using Biuret reagent by adding a standard solution of Bovine Serum Albumin (BSA) protein. The sample cuvette was prepared with a PAG and 2.5 ml of the Biuret reagent. The standard cuvette as filled with 0.05 ml BSA and 2.5 ml of the Biuret reagent. The blank cuvette was prepared by adding 2.5 ml Biuret reagent and 0.05 ml of distilled water. Three cuvettes were left for 30 minutes and color intensity was read by Bausch Lomb Spectronic Spectrophotometer at a wavelength of 540 nm.

Specificity test of PAG with Western Blot

Western blot was carried out by using fragments of PAG bands which had been run in SDS-PAGE and were transferred to the nitrocellulose membrane. The membrane was blocked with 3% BSA in 20 mM Tris-HCl at pH 7.5 and 150 mM NaCl for one hour then was incubated with the primary antibody (anti-PAG) diluted in Tris/NaCl containing 1% BSA. After washing with Tris-Cl containing 0.05% TWEEN 20, the membrane was incubated with secondary antibodies (anti-rabbit IgG labeled AP, 1:1000 dilution) and was added Western Blue Substrate (Promega, USA) (Aulanni'am, 2004).

Statistical analysis

The standard protein curve was made to obtain the molecular relative mass of the samples (Gaspersz, 1995). The relative molecular mass of each protein defined by data converted from relative migration distance (*Rf*) values according to following linear equation: Y = b0 + b1X; where Y is molecular weight (kDa), b0 is a constant, b1 is coefficient of relative migration, and X is relative migration of protein band.

The total protein concentration of PAGs by Biuret method was calculated as follows: $Y = 5.10^{-5}X$ Where Y is absorbance and X is a concentration of protein (µg/ml). The data of the Biuret method was statistically analyzed using ANOVA multivariate. Data were analyzed using SPSS version. 17.0 software (SPSS Inc, USA). A $p < 0.05$ were regarded as statistically significant.

RESULTS

The profile of the PAGs isolated from blood serum of Etawa crossbred goats using SDS-PAGE are shown in figure 1. The protein molecular weight was measured by relative migration when the protein passes through the separating gel (Figure 2). Then, based on the logarithmic equation (y = 2.401 + -1.4752X; R2 = 0.9829) obtained by calculating the relative migration, the molecular weight was obtained as a band that appeared on electrophoresis. The molecular weight of protein bands from six samples are presented in table 1. The serum of control and pregnant goats has the same protein profile. However, in blood serums of pregnant goats, there was a protein with a molecular weight of 55.85 kDa which was expected to be a specific PAG.

To ensure that the electroelution protein was a PAG, the elution results was examined by Biuret method to determine PAG protein levels. The results of the examination using the Biuret method can be seen as isolation of PAG in table 2. The protein concentration was the lowest in non-pregnant goats (1.83±2.98), then 3 months pregnant (3.79±2.72) and showed the highest value in 4 months pregnant (4.36±2.63). The indicated correlation in blood serum had a significant difference ($P<0.05$).

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Specificity tests were carried out to ensure that the detected protein was PAG. The results of the Western Blot test showed purplish bands on nitrocellulose membranes with a molecular weight of 55 kDa (Figure 3). This finding proved that the visible bands were PAG molecules. The molecular weight can be read using a reference marker protein with a molecular weight range of 15 to 260 kDa. Protein band with a molecular weight of 55 kDa was found in samples of pregnant goats in gestational age 3 and 4 months, whereas in samples of non-pregnant goats there was a protein band with a molecular weight of 23 kDa.

Table 1. Molecular weight of protein obtained from blood serum of Etawa crossbred goats

Serum Sample	Protein Molecular Weight (kDa)											
	15.29	**20.23**	**24.10**	**31.89**	**39.35**	**48.55**	**55.85**	**62.04**	**82.10**	**120.68**	**165.39**	**197.03**
Non-pregnant	+	+	+	+	+	+	-	+	+	+	+	+
3 month pregnant	+	+	+	+	+	+	+	+	+	+	+	+
4 month pregnant	+	+	+	+	+	+	+	+	+	+	+	+

(+) = positive band; (-) = Negative band

Table 2. The average concentration of PAGs isolated from blood serum of pregnant Etawa crossbred goats by using Biuret method

No.	Sample	Absorbance 1	Absorbance 2	Average concentration (µg/ml)
1	non-pregnant	1.48	2.18	[a] 1.83±2.98
2	3 month	5.22	2.36	[b] 3.79±2.72
3	4 month	2.45	6.23	[c] 4.36±2.63

Different superscript letters indicate significant differences ($p<0.05$); PAG: Pregnancy-Associated Glycoproteins; Absorbance was read in a spectrophotometer at a wavelength of 540 nm

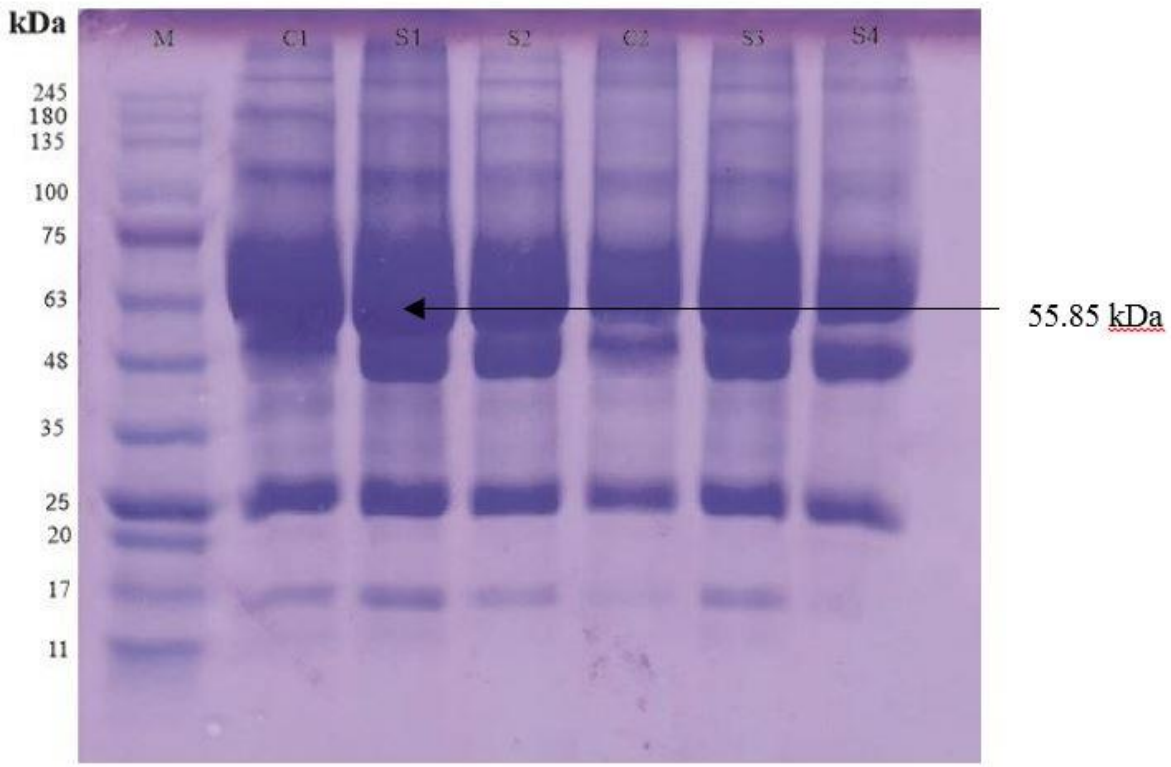

Figure 1. This is the SDS-PAGE analysis of PAGs isolated from blood serum of Etawa crossbred goats. Lane M: Tris-Glycine 4-20%, 11-245 kDa as marker; Lane C1 and C2: non-pregnant; Lane S1 and S3: 3 months pregnant; Lane S2 and S4: 4 months pregnant.

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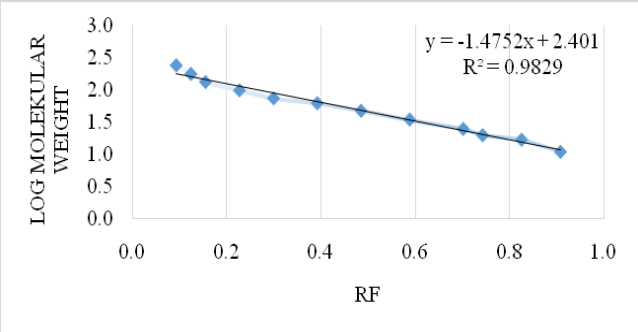

Figure 2. Determination of molecular weight by calculating the relative migration

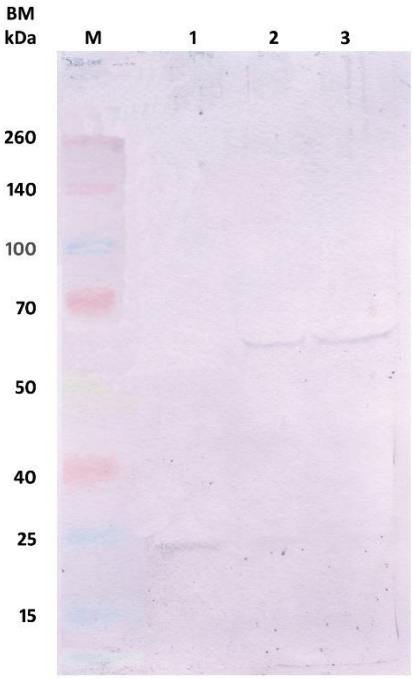

Figure 3. The Western Blot analysis for PAG isolated from blood serum of Etawa crossbred goats. Lane M: marker; Lane 1: non-pregnant; Lane 2: 3 months pregnant; Lane 3: 4 months pregnant.

DISCUSSION

According to the obtained results in the present investigation, the molecular weight of PAG in Etawa crossbred goats was 55.85 kDa. This finding is in accordance with the discovery of caprine PAG in the previous study by Amiri et al., (2004) that identification protein was performed by SDS-PAGE and found the molecular weight of caprine PAG was between 55 to 66 kDa. Moreover, Garbayo et al. (1998) reported different molecular mass (55 kDa, 59 kDa, and 62 kDa) for caprine PAG. PAGs can be detected in the maternal blood circulation from embryo implantation (Gordon, 1999). Trophoblast placental cells are present in blood circulation during implantation until parturition and are responsible for producing PAGs throughout the gestation period (Gonzales et al., 2000).

The protein concentration of PAG increases progressively at 3 and 4 months of gestation. Blood PAG levels steadily increased during early pregnancy in goats (Singh et al., 2019). Ispierto et al., (2016) reported PAG concentrations were significantly higher in twins compared to single pregnancies. The current research is in agreement with one of the statements by Cavanagh (1996) who mentioned PAG was first discovered as a substance-related to pregnancy and was detectable in 6-24 hours after conception in all species such as rats, humans, pigs, and sheep. Duplants (2000) declared that PAG was detected after the implantation and remained in the pregnant goat until parturition and disappeared after the birth process. Many factors influence the concentration of PAG, such as breeding (Ranilla et al., 1994; Guilbault et al., 1991), the number of fetuses (Benitez-Ortiz, 1992) and *in vitro* culture period (Ectors et al., 1996). Therefore, differences in PAG expression observed in the present study can be related to variations in breeds, procedures, and geographical location.

The results of present study showed the protein bands on nitrocellulose membranes, indicating a specific bond between PAG antibodies and PAG antigens isolated from Etawa crossbred goats pregnant. The further production of PAG increases the bond between PAG antibodies and PAG antigens and provides thicker protein bands. This finding is supported by Aulanni'am (2004), in Western Blot method, PAG antibodies recognize PAG antigens as specific antigens and bind together thus purplish-colored protein bands become visible.

CONCLUSION

The present study characterized PAG with a molecular weight of 55.85 kDa in Etawa crossbred goats at 3 and 4 months before parturition. Moreover, application of PAG as a biomarker of pregnancy was confirmed in Etawa crossbred goats.

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DECLARATIONS

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Consent to publish

All authors contributed to write and publish manuscripts in the World's Veterinary Journal.

Competing interests

The authors declare that they have no competing interests.

Author's contribution

IKN wrote the manuscript and conducted the research, TDL conceptualized the research, and HAP revised the final form of the manuscript.

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