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Production of Polyclonal Antibodies against Pregnancy Associated Glycoprotein in Cattle

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ABSTRACT

Pregnancy-associated glycoproteins (PAGs) are considered to be a large gene family in the trophoblasts of ruminants. PAGs determination precisely, PAG-1 in serum has been as the solid ground for pregnancy diagnosis in cow. Unluckily the PAG-1 and the antigenically related PAGs show a long half-life in serum less than 8 days and can be assessed 80 to 100 das postpartum, thereafter giving false results in cows bred within 60 days of calving. This study involves using novel polyclonal antibodies after immunizing rabbits and purification of the produced antibodies. The cattle are pregnant when the PAG hormone level is measured in a high level in a serum sample from the animal. It could give a precise method of detecting pregnancy at the early with few false positive results.

Key words: PAG, Polyclonal antibodies, Early pregnancy, Cattle.

INTRODUCTION

Pregnancy diagnosis is a highly needed issue in good reproductive monitoring, especial in the dairy farms (Oltenacu *et al*., 1990), when artificial inseminations fail. A reliable simple pregnancy testing for cattle has long been used. Several measures such as a milk progesterone assay, rectal palpation, estrone sulfate analysis ultrasound, and blood tests. The progesterone in milk assay is the most cost effective for the owner. Afterward the best is rectal palpation, (Oltenacu *et al*., 1990).

Though the prior measures for pregnancy diagnosis are really beneficial, all have not been practical. For example, assessment of progesterone around days 18-22 yield insufficiently elevated proportion of false positives. The existence of estrone sulfate in samples delivers another test, which is only effective after day 100 when concentrations increase. Detection of early pregnancy to achieve breeding. Development of alternate, cheap, assay is highly important.

Immunogenic assays are the best way to use in order to monitor a programmed breeding.

Immunogenic detection of pregnancy depends on specific antigens secreted by specific cells in the uterus of the cattle. Once the blastocyst implants into endometrium, the proteins pass to the blood (Jainudeen and Hafez, 2000). Which is considered as a pregnancy indicator (Hafez, 2000; Ball and Peters, 2004; Bearden *et al.,* 2004). PAG is an immunogen (Zoli *et al.,* 1990 a,b; Xie *et al*., 1991; Klisch *et al.,* 2005). Immunogenic proteins, when injected into the lab animals evoke the immune system to produce specific antibodies (Abbas *et al.,* 2000; Gordon, 2004). We aim to produce the antibody against PAG hormone for further application in immunogenic assays.

MATERIALS AND METHODS

Preparation of PAG for immunizations:

We have developed tissue culture systems for bovine trophoblast cells isolated from cattle and proliferation accomplished in Dulbecco's modified Eagle's culture medium supplemented with FCS. The cells exhibit the ability to express placental pregnancy-associated glycoprotein-The earliest stage placental tissues (trophoblast) were cultured in pools. These samples were collected from days (24 to 34) after slaughter in a local slaughterhouse.

Also, PAG were from tissue cultures of cotyledons obtained from uterus day 80 and day 150 cattle.

Cotyledon were gently separated from caruncles K and K the K cut K into V pieces 2 mm^3 each, washed K three K times in KDMEM (Sigma, St. Louis, MO, USA) which contains penicillin 100 U/mL, streptomycine100 mg/mL and fungizone (0.5 mg/mL); and cultured in the same medium at 37°C ine5%CO_2eair for 12 h. After the incubation, the medium was collected by centrifugation, and the supernatants stored at -20°C until used. The supernatants were collected after 12 h incubation then

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centrifuged to remove cell debris and stored at -20ºC for further steps of precipitation and purification of the hormone to be ready as an immunogen for immunize rabbits. Extraction of the hormone was done directly from the tissue as well.

Precipitation of the PAG hormone

Dry ammonium sulphate ((NH4)2SO4) was slowly added to the supernatant separately to obtain a 40%-saturated solution. Proteins were allowed to precipitate for 4h and centrifuged at 10,000 g for 1h. The pellets were discarded and additional ammonium sulphate was similarly added to the stirred supernatant to achieve 80%-saturation followed by centrifugation.

The pellets were retained and resuspended in 0.01 M Tris- HCl buffer (pH 7.6). The resuspended pellet was dialysed 3- 4 times in the same buffer using dialysis bag (cut off value >2 kDa) and concentrated by sucrose. The dialysed and concentrated solutions were stored for further use. The uterine fluid proteins were precipited with ammonium sulphate. Dry ammonium sulphate (608 g/ L) was slowly added for 5-10 minutes with continuous stirring to obtain 85% saturated solution. Proteins were allowed to precipitate for 2 h and then centrifuged at 10,000 g for 1 h. Supernatant was concentrated similarly as above. The dialysed and concentrated solution was stored for further pellet was concentrated similarly as above. The dialysed and concentrated solution was stored for further and resuspended in 1-2 pellet volume of PBS. The precipitate was dialysed 3-4 times in PBS buffer and concentrated similarly as above. The dialysed and concentrated solution was stored for further use.

Purification of the PAG hormone

By using column containing 100 mL of bededepep statin-A agarose 4% (Sigma) equilibrated in the buffer. The column was washed (10ecolumn volumes of loading buffer; then 10 column volumes of 20 mM Tris pH 7.0, 1 M NaCl, 1% Triton X-100, 1 mM EDTA, 0.02% (w/v) sodium azid, 20 mM PMSF, 1 mM 2-mercapto-ethanol). Were eluted by increasing the pH. Elution fractions were collected and pooled and their protein concentration measured by Qubit® Protein Assay Kit.

Estimation of protein

The concentration of protein in all the biological samples were determined by using Qubit® Protein Assay Kit.

Rabbits immunization and Preparation of polyclonal antibodies against PAG

The dose of PAG which used an antigen for immunization is 500 µl, containing 1000 µg for each rabbit. This dose is in agreement with the procedure used by Hudson and Hay (1989). The dose of hormone immunization in the rabbit is around 50-1000 µg. The production of anti-PAG antibody was organized on five male rabbits aged 2 months. Firstly, immunized on the day-0, inoculating subcutaneously 1000 µg of PAG, reconstituted in 500 µl PBS, emulsified in an equal volume of complete Freud's Adjuvant (CFA) (Sigma), injected at multiple sites S/C (Singh *et al*., 2003). (Table 1). Each rabbit injected with 500 µl of (Phosphate Buffer Saline) diluted in 500 µl CFA as control. Before immunization, -ve control blood was collected from each rabbit. The first booster injection was given at 2 week interval with Freund's Incomplete Adjuvant (Sigma) and subsequent boosters were given in the same manner at 2 week interval. Bleeding was done after sixth booster and sera were collected, stored at – 20ºC in small aliquots until used.

Screening of the hyper immunesera

High binding ELISA plates were coated with PAG from (GenScript®) antigen in carbonate bicarbonate buffer (coating buffer) in concentration of (50µg / 100µl /well). The plates were incubated at 37ºC for 1 hour and left overnight at 4ºC. Unbound antigen solution was aspirated and the unbounded sites on the coated wells were blocked using blocking buffer (1% BSA). 100 µl of blocking buffer per well were dispensed in the coated 96 microtiter plates followed by incubation for one hour at 37ºC. Serum from each rabbit was diluted double fold serial dilution. The coated wells were inverted and flipped several times to decant the samples, then the plates were washed 3 times using 300µl/well washing buffer (PBS + Tween 20 (0.05 %) 5 minutes each on a horizontal shaker for removal of the unbounded protein. The plates were washed as in step 3. Enzyme-labeled goat anti-rabbit-Ig peroxidase conjugate at the predetermined dilution (1/4000) was added (100 µl/well) and incubated for one hour at 37ºC. The plates were washed as in step 3. The ABTS enzyme substrate was added (100 µl per well) and incubated for 30 min at 37ºC until color develops. 100 µl of the 1% SDS stopping buffer were added/well and then the plates were assessed using ELISA reader. Cut off value was equal to the mean OD of negative samples plus 2 standard deviation.

Separation of anti-PAG antibodies

Immunoglobulins were precipitated from the hyper immune sera as per the procedure described by Hudson and Hay (1989). Saturated ammonium sulphate solution was prepared by dissolving 1000 g of ammonium sulphate in 1 L distilled water at 50ºC and pH was adjusted 7.2 with dilute ammonia solution. Serum was diluted 1:2 with saline and saturated ammonium sulphate solution was added till reaching a conc. of 45% saturation (v/v). Solution was stirred at room temperature for 30 minutes and centrifuged (1000g, 4ºC, 15 minutes). Precipitate washing was done with 45% saturated ammonium sulphate and then repeated centrifugation. Precipitate then was re-dissolved in the as the volume of PBS exactly. A concentration of 40% ammonium sulphate was used to re-precipitate the immunoglobulins. Precipitate was centrifuged and washed again then centrifuged. Finally, the precipitate was dissolved and dialyzed 3-4 times against PBS at 4ºC to get rid off the salt. Concentration of immunoglobulins was estimated by determination of absorbance at 280 nm using spectrophotometer.

Purification of anti-PAG antibodies

Affinity chromatography was carried out by using NAb™ Spin Kits, 0.2mL for Antibody Purification (Thermo Fisher SCIENTIFIC).

RESULTS

Preparation and purification of PAG

The PAG hormone was prepared from early placental tissues, precipitated by ammonium sulphate and purified using affinity chromatography. The protein content was estimated to be ready for rabbit immunization.

Immunization and screening of the hyper immunesera

It evidences that the pre-immune lab. animal without PAG immunization have O.Ds values very low, compared with other animals after immunization by PAG. Before immunization with PAG (control negative) has no anti-PAG antibodies present in the serum, Anti- PAG atibodies titer highly elevated after booster II.

The four tested rabbits gave a high titre of 1/1280 which shows that good immunization has been accomplished.

Separation of polyclonal antibodies

Saturated ammonium sulphate solution was used for precipitation of the antibodies following that the purification step which is an important stage to guaranty the purity of the produced antibodies using column chromatography.

Table 1: The immunization protocol

Dose No.	Day	Adjuvant	Dose and Route
First	0	CFA	1 ml S/C
Second	15	IFA	1 ml I/M
Third	30	IFA	1 ml I/M
Fourth	45	IFA	1 ml I/M
Fifth	60	IFA	1 ml I/M
FINAL	4 days before serum collection	Antigen only	1 ml I/M

DISCUSSION

Infusion of PAG hormone to bunnies has actuated immune response creation reaction (Anti- PAG). Immune response (against PAG) has expected both PAG principles. The most elevated enemy of PAG titer was gathered from hares.

Immune response creation incorporates the utilization of lab creatures, for example, hares, mice and guinea pigs (Aulanni'am, 2005). These lab creatures can make 25 ml, 100-200 µl and 1-2 ml serum to be gathered. This examination utilized bunnies, so as to create plentiful serum, simple to deal with and low coast, likewise, safe to test commonly through bleeding.

The creation of against PAG antibodies in the bunnies could be explained on the hypothesis that the immune response is the protein that delivered by plasma cells since there is an association between the lymphocyte B and the presence of the antigens (Abbas et al., 2000; Tizard, 2004). Antibodies against some antibiotic resistant bacteria also had been evaluated immunologically and by molecular methods in previous studies (Hossam *et al.,* 2016; Elhariri *et al.,* 2017; Abdel-Moein *et al.,* 2017).

The procedures could be preceded in short as follows: PAG protein went into the lab creatures and afterward being handled in the Antigen Presenting Cells (APCs), at that point exhibited to the receptors of the T cells, associated by significant histocompatibility complex (MHC) class I and II. APCs produce and discharge cytokins, animating the T cells to multiply and separate. At that point, following the chain resistant response, the T cells were animated to discharge their own cytokins, which actuated B cells on three levels: initiation, multiplication, and separation into plasma cells, which produce Ig (immunoglobulin). The T cells receptors is extremely careful to the setup of specific proteins/MHC and each T cells just has a receptor kind to ensure the safe reaction is precisely explicit. Plasma cells at that point produce hostile to PAG antibodies. As indicated by Tizard (2004), immune response titer post supporter II is raised than post promoter I, in light of the fact that the creature body has memory to perceive antigen which has been presented to previously (Xie et al., 1991).

Conclusions

Protein created by trophoblastic cell of blastocyst, named pregnancy-related glycoprotein (PAG), is one of protein which exists in pregnant cattle serum can animate counter acting agent when infused to lab animals. One of the early pregnancy recognition systems depends on invulnerable reaction, utilizing the Ag - Ab response. Anti-PAG, got from vaccination, is relied upon to be a device marker in discovery of early pregnancy in cows and can be applied in numerous indicative tests.

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