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Research Article

# Development and Validation of a Solid Phase Radioimmunoassay System for the Determination of Pregnancy-Associated Glycoproteins in Bovine Serum

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## Abstract

The Pregnancy-Associated Glycoproteins (PAGs) belong to aspartic protease family and are synthesized by trophoblast cells of ruminant placenta and secreted into maternal blood and milk. These biomarkers very closely associated to pregnancy have demonstrated their efficiency and reliability in detection of vital embryos or pregnancy loss. In order to measure the concentration of PAGs in bovine serum, a direct radioimmunoassay (RIA) system was developed and validated. The radioiodinated bPAG tracer was prepared by chloramine-T direct method and the solid phase was used in RIA/PAGs as a separation system. The optimization tests allowed to set the assays procedure as working buffers, time and temperature of the assays incubation. Assay sensitivity was 0.63 ng/mL, the intra and inter-assay CVs were 7.7% and 5.3% respectively and the accuracy of the assays was expressed by a good parallelism and recovery tests. The serums bPAG values determinated by this developed system were consistent with the reproduction status and pregnancy stages of cows. The developed RIA/PAG system can provide the laboratory with an efficient method for the accurate detection of pregnancy, which could offer veterinarians and farmers an important tool for the reproductive management in dairy herds. Moreover, this diagnostic tool can also be developed for other livestock species.

**Keywords:** Bovine pregnancy-associated glycoproteins, Radioimmunoassay, Serum, Solid phase, Validation

## Introduction

Reproductive performances and efficiency in dairy herds are highly dependent on accurate and timely diagnosis of non-pregnant females [1,2]. Early detection of non-pregnant cows reduce the intervals between calving and consequently optimize milk production [3]. To identify pregnancy, among the most commonly used existing biomarkers [3,4], apart from conventional techniques [5-8], Pregnancy-Associated-Glycoproteins (PAG), since they were isolated and purified in ruminants, have revolutionized pregnancy monitoring. PAGs belong to the family of aspartic proteinases [9] and are synthesized by the trophoblast cells of the placenta of ruminant, then secreted into maternal blood circulation 20 to 22 days of gestation in goats and ewes, 30 days of gestation in cows and doe [4,10]. The presence of the proteins has also been determined in milk and enabled their use to detect embryonic and fetal mortalities [11,12]. PAGs concentrations in milk are 10 to 50 times lower than those in plasma [13] and 1.5% of the amount present in serum [14]. The quantification of the glycoproteins has been developed in different species [13], by immunological techniques, depending on the availability of equipement in each laboratory, such as RIA [15] and ELISA [14-16]. The PAG-RIA and PAG-ELISA tests had similar accuracies for the diagnosis of early pregnancy in dairy cows at day 28 after insemination [5]. Considering the economic impact of the quantification of PAGs on the management of reproduction in dairy farms, in-order to provide users with a diagnostic tool for pregnancy, to make the kits easily available in our country and to allow the expansion use of the technique, a direct, fast and reliable in-house RIA kit to measure PAGs has been developed. The present study describes the steps for preparing a basic reagents of PAG/RIA kit, as a purified radioiodinated bPAG tracer, solid-phase separation system and a range of standards, as well as the optimization of the assays and the analytical and clinical validation of the kit in bovine serum.

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# Material and Methods

## Ethical Approval

All the animal studies were conducted with the utmost regard for animal welfare, and all animal rights issues were appropriately observed. No animal suffered during the work. All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

## Reagents

Purified bPAG 1 (boPAG67kDa) and Rabbit Antiserum raised against caprine PAG 55+62 kDa (crude antiserum R#706) were purchased from Pr J.F Beckers and delivered by Bioproduct Consult SPRL (Belgium). $^{125}I$ as sodium iodide was supplied by Isotop (Hungary). Chloramine-T and Sodium-Metabisulphite were from Sigma Aldrich (Germany). All other reagents used in this study were of analytical reagent grade and were obtained from Fluka and Sigma Aldrich.

## Preparation of Radioiodinated PAG Tracer

### *- Radioiodination of PAG Glycoproteins*

Iodination of bovine PAG was carried out under a radiochemical hood, using the direct method of Hunter and Greenwood [17], already presented in a previous work [18] with some differences. Briefly, 10 µL of bPAG (10 µg) in phosphate buffer solution (0.1 M, pH:7.4) was mixed with 4 µL (688 µCi) of $^{125}INa$. The Chloramine-T was added and stirred slightly for 1min at room temperature then the reaction was stopped by addition of Sodium Metabisulfite. 100 µL of potassium iodide (KI) are added to the mixture in order to facilitate the separation between the tracer and the free iodine during the purification step. The labeling activity was measured in an ISOMED activity meter (Capintec).

### *- Purification of the Tracer*

The purification of the tracer was carried out by gel filtration on a glass column (33×1.5 cm) of Sephadex G-75, equilibrated and eluted with a phosphate buffer/BSA (0.1 M, pH 7.4, 1% BSA. 0.1% sodium azide). The radioactivity of an aliquot (10 µL), of each fraction collected at a flow rate of 1 mL/tube, was counted in NaI (Tl) scintillation counter (Perkin Elmer). The fractions corresponding to the peaks were pooled and evaluated for radiochemical purity (RC purity) and immunoreactivity. The obtained tracer was stored in aliquots of 150 to 200 µL, in the refrigerator (8°C) and in the freezer (-6°C).

### *- Evaluation of the Tracer*

*Radioiodination Yield*: The radioiodination yield of the tracer $^{125}I$-bPAG produced was evaluated by using the elution profile obtained by purification of the tracer. It represents in percent, the ratio of the activity associated with the molecule (µCi) and of the total activity (µCi).

*Radiochemical Purity and Immunoreactivity:* The radiochemical purity of the selected fractions was evaluated by paper electrophoresis and the specific and non-specific binding (NSB) binding rates were performed in duplicate. They were evaluated as presented previously [18].

*Specific Activity:* The specific activity of the tracer, estimated from the elution profile, was evaluated by the ratio of the activity of the tracer (µCi) and 10 µg of labeled PAG (assuming that the total amount of PAG used has been labeled)

### *- Stability of the Tracer*

The stability of the tracers produced ($^{125}I$-$bPAG_{67KDa}$) was studied over a time (8 weeks) and a temperature of storage (-6°C and 8°C), by estimating the radiochemical purity (PRC%) and the specific binding (Bmax%), and non-specific binding (NSB%) of tracer.

## Preparation of bPAG Standards

Bulk solution (1 µg/mL) of pure stock $boPAG_{67KDa}$ (lyophilized powder) was prepared by dissolution of 10 µg of boPAG in 10 mL of a young heifer serum (PAG-free serum) to minimize the matrix interferences, containing as conservator agent, sodium azide (<0.1%). Working standards, covering the concentration range from 0.25 ng/mL to 100 ng/mL were prepared. The standards were aliquoted (1 mL) in vials, then lyophilized and stored at 10°C. The $boPAG_{67KDa}$ working standards used in RIA assays were prepared by reconstitution of the lyophilized standards in nanopure water.

## Purification of Antiserum and Preparation of Solid-phase

### *- Purification of Antiserum AntibPAG*

Rabbit polyclonal antiserum PAG (Crude antiserum Anti-caPAG (55+62kDa): AS#706, PAG accession numbers P80935) was purified to obtain the globulin fraction by using the sodium sulphate precipitation method [19]. The antiPAG precipitate obtained was dissolved in a 0.01 M phosphate buffer, pH 7.4 then dialyzed overnight in a tubing (Spectra/Por Biotech), against a 0.005 M phosphate buffer pH 7.4. The obtained globulins after dialysis are stored in a glass flask, lyophilized, and stored at -6°C.

### *- Preparation of Solid Phase*

Separation procedure used in the RIA assay was the solid phase system [20]. In the present assay, the purified rabbit PAG antibody was diluted to different titers (1/100-1/80000) and coated as procedure described previously [18]. The stability of coated tubes was studied over nine months

by estimating the specific binding rates (Bmax %) in titer of 1/40000. The tubes intended for non-specific binding (NSB) evaluation, were prepared by the same procedure, but the antibody was replaced by a buffer.

### Optimization of Assays Parameters

The optimization of the developed kit was carried out by evaluating assay parameters related to assay procedure, such the assay buffer systems (phosphate and Tris HCl buffers), the incubation condition of assays (duration time: 1-24 h, temperature: 6°C, 22-25°C (room temperature) and 37°C and the number of washes of the assay tubes after decantation.

### Radioimmunoassay Procedure of PAG

The assay involved 50 µL of standard/sample and 250 µL of tracer (25000-35000 cpm), added into in-house coated tubes (solid-phase system with 1/80000 antibody titer). The contents of the tubes were mixed and incubated overnight at room temperature, under shaking. Finally, the tubes were decanted, washed once with 500 µL/tube of washing solution and the radioactivity of each tube was measured for 1 min. the displacement curve of RIA inhibition standards has been plotted on the y-axis, in a linear scale of binding rates B/Bo ratio (ratio between the amounts of radiolabeled PAG bound to antibody, in presence and in the absence of unlabeled PAG) and on the x-axis, in a logarithmic decade of the standards PAG concentration.

### Validation of PAG Radioimmunoassay

Several quality control parameters of the assays curves were calculated automatically by the LBIS software used with the LB gamma counter. it concerns the sensitivity of an assay, defined as the minimum detection limit (MDL) or the mean concentration minus three times the standard deviation of Bo and the expected concentrations at 80%, 50% and 20% of B/Bo intercepts. The reproductibility of the RIA assay was carried out using two serum samples taken from pregnant cows. It is determinated by calculating the intra- and the inter-assay coefficients of variation (CV%), respectively 8 times whithin the same assay and in five consecutive assays. The accurancy of the RIA test was assessed by parallelism, which was determined by evaluating the serial dilution of pregnant bovine serums containing high concentrations of PAG with PAG-free serum and by a recovery test, determinated by adding increasing concentrations of purified boPAG$_{67KDa}$ (5, 50 and 100 ng/mL) to bovine serum, containing known PAG concentrations (0.93 and 5.6 ng/mL). The calculated recovery rate was the percentage ratio between the observed and expected values (ng/mL). For the clinical validation of RIA assay, the serum of PAG concentrations was determinated in 17 dairy cows, including 5 non-pregnants cows and 12 cows at differents stages of gestation. Pregnants cows were monitored by veterinarian from insemination until confirmation of pregnancy by transrectal palpation. One blood sampling (10 mL) was taken from the jugular vein in dry tubes, the serum was separated by centrifugation at 3000 g for 15 min, and stored in freezer until assay. After one assay, the samples with higher PAG concentrations (>100 ng/mL) were diluted than re-assayed to approximate the true values. All assays were performed in duplicate.

To perform the clinical validation, the bPAG assay results determined by the developed kit had to be compared with those determined by a commercial kit, however, a problem in acquiring the commercial kits forced us to compare our results with the clinical informations and the events recorded by the veterinarian on the individual cow records during these inspections.

### Data Analysis

Statistical analysis was performed by MS Excel and ANOVA test from XLStat statistical software. Statistical significance was considered at the $P<0.05$ level.

## Results

### Radioiodinated Tracer Quality

An example of the elution profile obtained by the purification of $^{125}$I-bPAG$_{67KDa}$ tracer was given in *Fig. 1*. It shows the presence of two peaks, eluted successively between fractions 16-19 and fractions 43-45. The first one with high activity corresponds to the tracer $^{125}$I-bPAG and the second one represents the free iodine. The radioiodination yields of $^{125}$I-bPAG determined from the elution profile was 80.76%. The specific activity of the radiolabeled tracer was 55.6 µCi/µg. The radiochemical purity was 97% and the maximum binding of labelled boPAG$_{67KDa}$ in presence of an excess of specific antibody was approximately 42.6%. The non-specific binding (NSB) was about 2%. The stability of the tracer $^{125}$I-bPAG$_{67KDa}$ recorded by radiochemical purety (RCP), showed

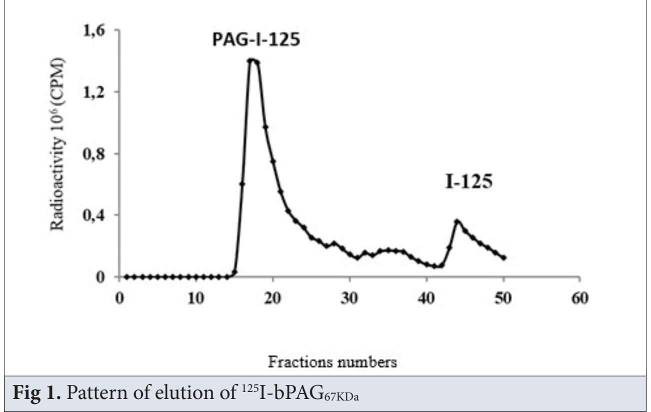


**Fig 1.** Pattern of elution of $^{125}$I-bPAG$_{67KDa}$

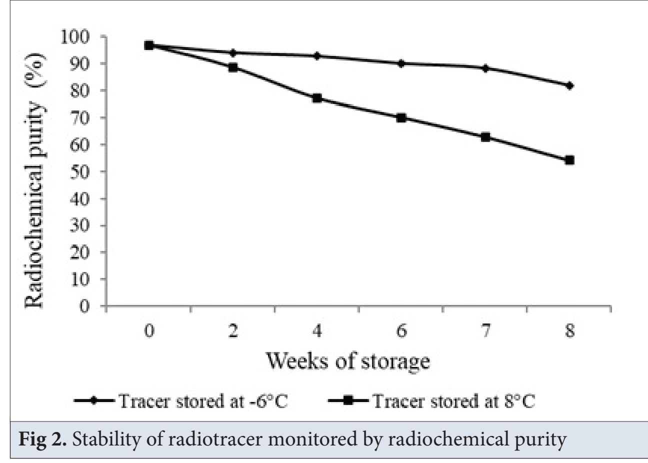


**Fig 2.** Stability of radiotracer monitored by radiochemical purity

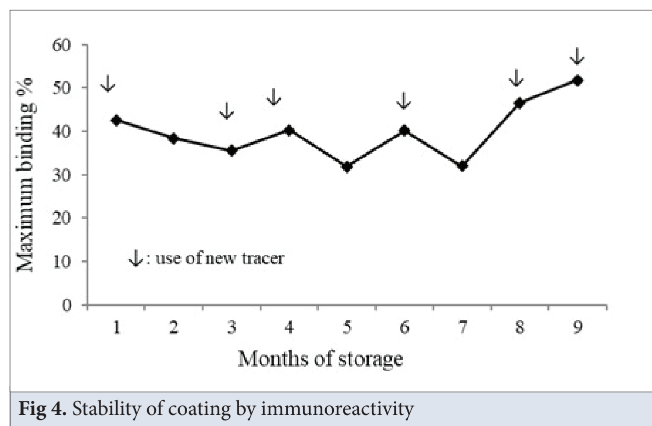


**Fig 4.** Stability of coating by immunoreactivity

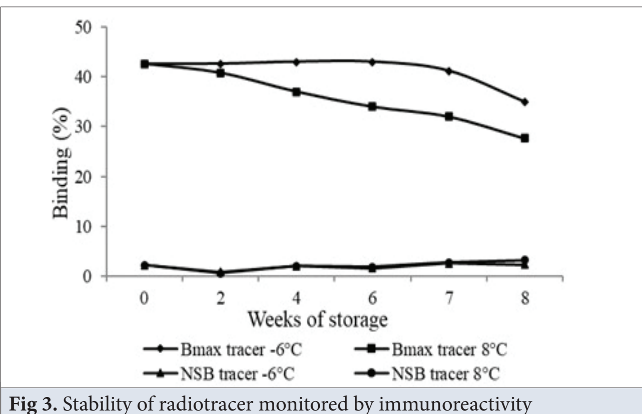


**Fig 3.** Stability of radiotracer monitored by immunoreactivity

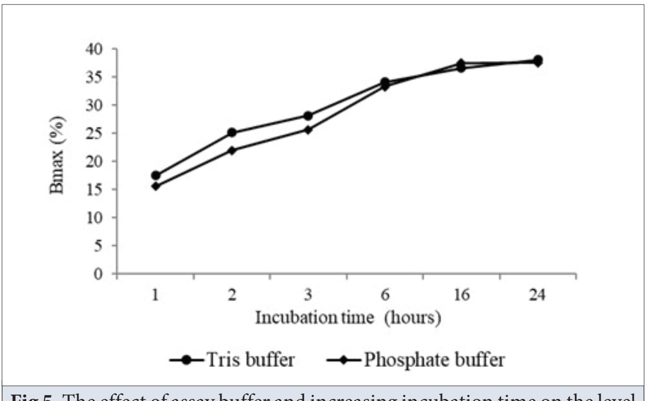


**Fig 5.** The effect of assay buffer and increasing incubation time on the level of specific binding

significant positif impact (P<0.05) of the storing tracer at a low temperature (-6°C) than 8°C *(Fig. 2)*, the means RCP rates obtained over entire storage period were 90.6±5.3% and 74.9±16.0% respectively. Concerning the storage time effect, the RCP values recorded for the two tracers (-6°C and 8°C), decreased very significantly (P<0.01) during the storage period, marking a stability between weeks 2-4 and 6-7. The degradation rate during the first month of storage was 4% and 20% respectively and it reached 16% and 44% throughout the storage period. The stability of the tracer recorded by specific and non-specific binding, significantly (P<0.01) confirms the interest of maintaining the tracer at a temperature of -6°C *(Fig. 3)*, the average values of Bmax obtained are 41.2±3.1% against 35.7±5.6% for the tracer stored at 8°C. As for the non-specific binding rates, the averages values recorded throughout the storage period of the two tracers (-6°C and 8°C) were respectively 1.9±0.6% and 2.1±0.9%. For the purposes of the RIA assays, the $^{125}$I-bPAG$_{67KDa}$ tracer produced was pooled and stored at -6°C.

## Solid Phase Quality

Stability assessment of the coated tubes, stored at a temperature of 4-8°C, performed over a period of nine months, showed no significant changes in immuno-reactivity results. The Bmax values obtained averaged 39.9±6.5%, varying from 32% to 52% *(Fig. 4)*, depending on the quality of tracers used to estimate the specific binding.

## Assay Procedure Optimization

During the implementation phase of the assay, two buffer assay system were tested combined with gelatin (0.2%), the first one was the phosphate buffer (0.05 M, pH 7.4) and the second was Tris HCl buffer (0.025 M, pH 7.2). The specific binding levels recorded following the use of the two buffers systems were significantly (P>0.05) identical at all incubation period *(Fig. 5)*. The values obtained after 16 h of incubation of the assay are 37.5% for the phosphate buffer and 36.6% for the Tris HCl buffer. The same *Fig. 5* showed a very significant increase (P<0.001) in specific binding levels with increasing the assay incubation time from 16.6% (1 h) to 37.1% (16 h), reaching a plateau up to 37.9% (24 h). Regarding to the incubation temperature, the specific binding rates after 16 hours of incubation at temperature of 37°C, 22-25°C and 6°C, showed a significant difference (P<0.05) *(Fig. 6)*, the means rates recorded were 34.5±1.2%, 37.0±1.1% and 31.8±1.0% respectively. The shapes of the competition of kinetics reaction over all the incubation time of the 3 incubation temperature tested were identical. Finally, the assay tube washing step recorded no significant difference

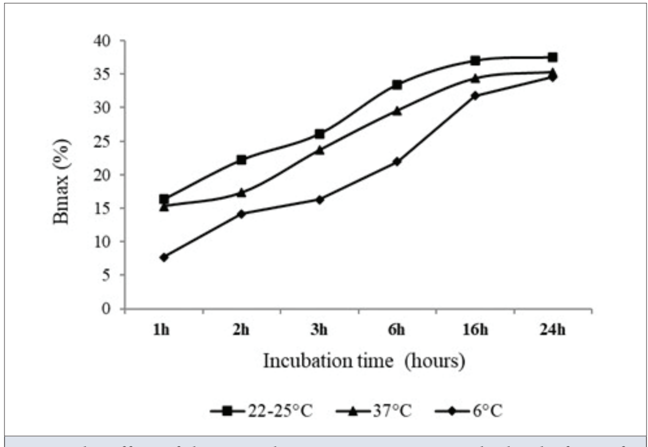


**Fig 6.** The effect of three incubation temperature on the level of specific binding

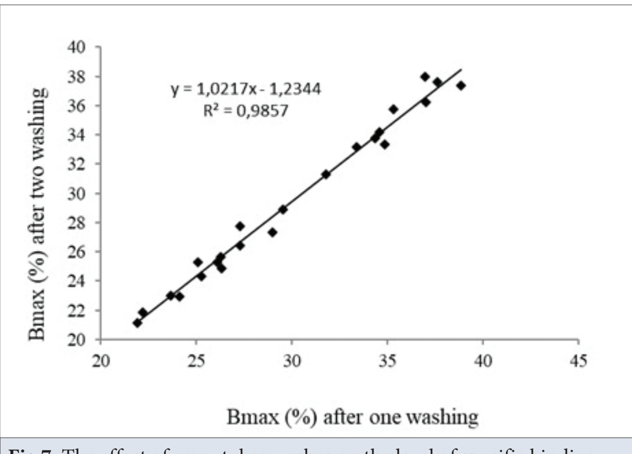


**Fig 7.** The effect of assay tubes washes on the level of specific binding

(P>0.05) in binding rates, when increasing tubes washes after decantation from one to two, the average rates recorded were respectively 29.9±4.8% and 29.1±5.0%. The maximum binding values obtained showed a good linear relationship between the two tested washes with a correlation coefficient $R^2$=0.99 and the regression equation of the following form y=1.02x-1.23 (n=23) *(Fig. 7)*.

## Development of PAG Radioimmunoassay

A typical displacement of standard inhibition curve of the RIA/PAG system obtained was showed in *Fig. 8*. The mean standards inhibition curve ranged from 96.11% to 8.4% binding when 0.25-100 ng of boPAG was added per assay tube. The quality control parameters of standard curves tested over 10 assays *(Table 1)* showed no significant change (CV<10%). The mean maximum binding rate of 22.0±1.8% was obtained with $^{125}$I-bPAG$_{67KDa}$ tracer and a final antibidy dilution of 1/80000. The nonspecific binding (NSB) value obtained was on average 2.4±0.2%. The developed RIA system can detect a wide range of PAG concentrations as shown by the expected PAG concentrations at 80%, 50% and 20% B/Bo intercepts, which were 1.5, 7.6 and 33.8 ng/mL respectively.

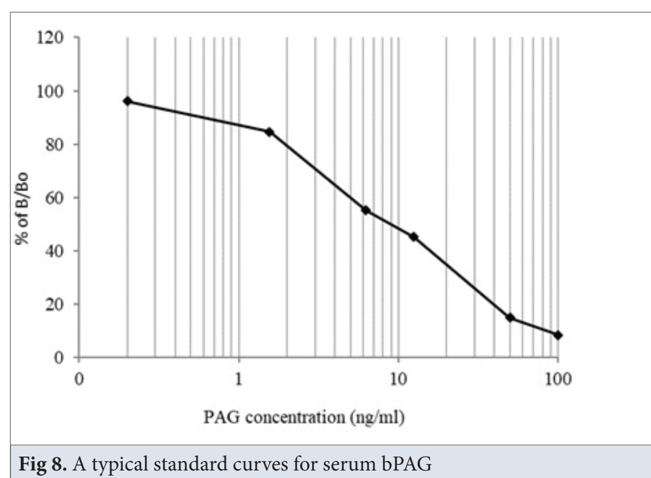


**Fig 8.** A typical standard curves for serum bPAG

***Table 1.*** *Performances of quality control parameters of a standard curves*

| Assay Parameters | Results (n=10) |
|---|---|
| Zero standard binding (%) | 22.04±1.79 |
| Non Specific Binding (%) | 2.43±0.15 |
| **Expected Doses (ng/mL) at:** | |
| 20%B/Bo | 33.83±1.66 |
| 50%B/Bo | 7.61±1.26 |
| 80%B/Bo | 1.54±0.43 |

***Table 2.*** *Properties of the direct RIA kit for measuring pregnancy-associated glycoprotein in serum*

| Parameter | Value |
|---|---|
| Sample volume (μL) | 50 |
| Measuring range (ng/mL) | 0.25-100 |
| Detection limit (ng/mL) | 0.63 |
| Dilution linearity (%) | 104.3 |
| Mean recovery rates (%) | |
| 0.93 ng/mL | 89.3 |
| 5.6 ng/mL | 85.2 |
| Intraassay CV (%) | |
| 1.3 ng/mL | 7.7 |
| Interassay CV (%) | |
| 26.3 ng/mL | 5.3 |

## Validation of PAG Radioimmunoassay

Validation properties of the direct RIA kit for measuring pregnancy-associated glycoprotein in serum was shown in *Table 2*. The minimal detection limit (MDL) of an assay was 0.63±0.20 ng/mL. The reproducibility of the RIA system (PAG$_{706}$) showed intra-assays and inter-assays CVs of 7.7% (1.3±0.1 ng/mL) and 5.3% (26.3±1.4 ng/mL) respectively. The accuracy of the assay was expressed by the good parallelism observed between the diluted bovine serum samples containing high concentrations of PAG and the curve of standards PAG ($R^2$=0.97, P<0.0009) *(Fig. 9)*

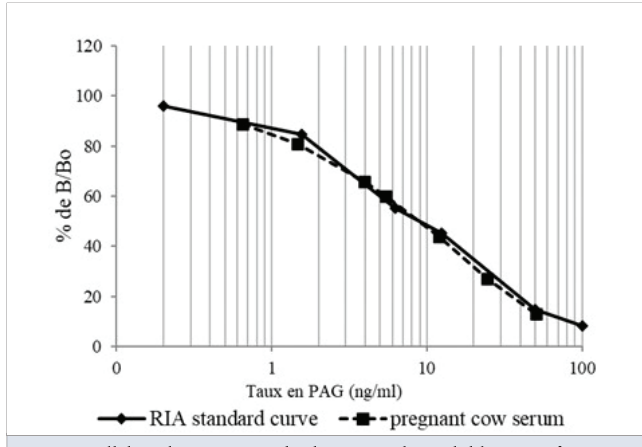


**Fig 9.** Parallelism between standard curve and serial dilutions of pregnant cow plasma sample.

***Table 3.*** *Mean (±SD) PAGs serum concentration of non pregnant and pregnant recorded cows*

| Physiological Status of Cows | PAG Concentration (ng/mL) |
|---|---|
| Non pregnant (n=5) | 0.69±0.32 |
| Pregnant (months) | |
| 1 (n=1) | 2.40 |
| 2 (n=3) | 10.88±1.99 |
| 3 (n=1) | 0.90 |
| 5 (n=1) | 0.60 |
| 6 (n=1) | 73.90 |
| 7 (n=2) | 133.99±3.23 |
| 8 (n=1) | 350.50 |
| 9 (n=2) | 597.33±153.12 |

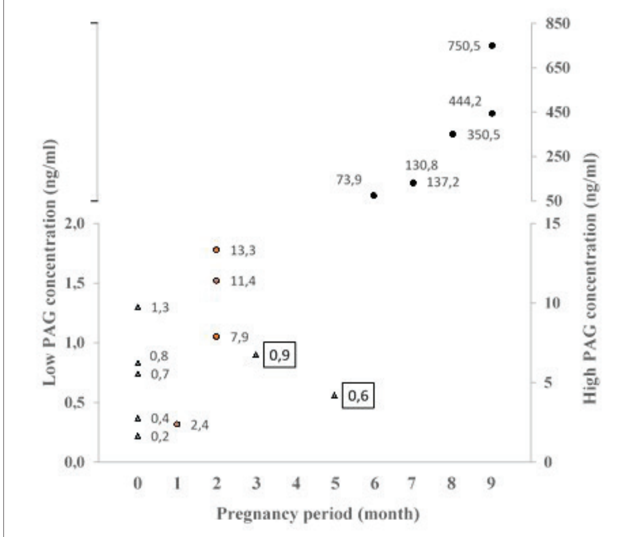


**Fig 10.** Serum PAG concentrations in various physiological status cows measured by in house RIA kit

and the satisfactory recovery of serial dilution of serum samples (104.3%) and mean recovery test of 89.3% (0.93 ng/mL) and 85.2% (5.6 ng/mL).,

The measurement of bPAG concentrations determined by the developed $PAG_{706}$ RIA system, in serum samples from 5 non-pregnant cows and 12 pregnant cows, showed values ranged from 0.22 to 750.45 ng/mL. For the non-pregnant cows, the average PAG concentration was of 0.69±0.32 ng/mL and for the pregnant cows, data revealed a constant increase of the PAG levels, depending on the month of pregnancy *(Table 3, Fig. 10)*. Two cows were sampled at the $3^{rd}$ and $5^{th}$ month of pregnancy, according to the veterinarian informations, recorded PAG concentrations of 0.9 and 0.6 ng/mL, respectively.

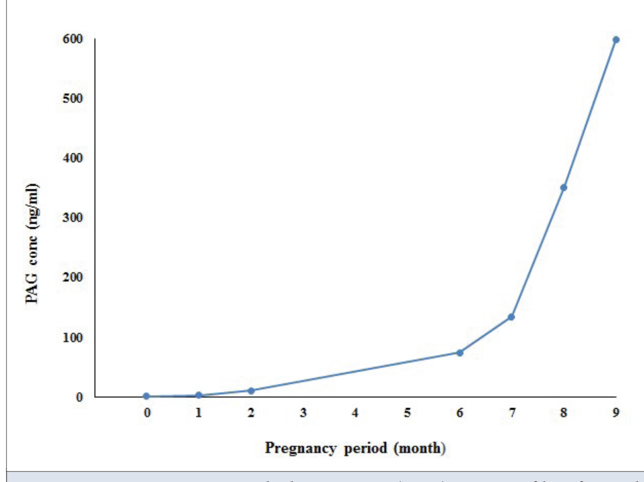


**Fig 11.** Pregnancy-associated glycoprotein (PAG) sera profile of tested cows during pregnancy

## Discussion

Although the determination of the concentration of Pregnancy-Associated Glycoproteins (PAGs) has proven useful for monitoring pregnancy in cattle and other dairy animals over the past 20 years [21], the kits of assays are still not available in some countries which do not always have the means and the sufficient conditions to regularly provide kits. As part of the development study of reagents kits for in-house RIA determination of Pregnancy-Associated Glycoproteins (boPAGs) in bovine serum, the first basic reagent prepared was a radioiodine tracer. The assessment of the quality of the obtained tracer reveals very satisfactory results in terms of radiochemical purity and stability over time when it was stored at -6°C. The specific activity of the tracer was in the range of the values obtained previsouly (60-80 µCi/µg), from tracers radiolabeled with 1 mCi [22], it is directly related to the low dose of Na $^{125}I$ used for radiolabeling the proteins, compared to those previously reported (2 mCi) [15]. Maximal binding values of labelled $boPAG_{67KDa}$ were similar to those previsiouly reported [15,23], with a different RIA system and the non-specific binding (NSB) values were remained below the ideal limit of 5%.

Despite the widespread use of the immunoprecipitation separation system (double or second antibody precipitation) in all previously reported RIA/PAG studies [15,23-25], in

the RIA/PAG system developed, a solid phase separation system has been used and was has proven effective and stable over time. The advantage of the separation system is that it does not involves the development of a second antibody, a long incubation and a separation step by centrifugation, for these reasons, it was the most commonly used [26]. In addition, the separation system greatly minimizes the matrix effect [27].

The purpose of this step was to set the optimum conditions allowing the immunocompetition reaction to reach equilibrium. In the developed RIA/PAG, the phosphate buffer assay system was retained on Tris HCl buffer, which is most commonly used in later RIA/PAG assays [9,15,23-25]. A single incubation, at room temperature, for 16 h was necessary. In all previous studies, in the reported RIA/PAG systems developed, the optimal time and temperature for incubation of the validated assay reaction was 16 h at 22-25°C for the preincubation period, then 2 h at 20°C for the incubation period with added tracer [2,15,22-25,28-30]. Decanted tubes were washed only one time, reducing non-specific binding (NSB) to insignificant levels [31].

Direct assays with heterologous RIA systems, based on antisera raised against the caprine PAG ($caPAG_{55+62kDa}$) immunogen and the pure bovine $bPAG_{67KDa}$ used as standard and tracer have been undertaken. Compared to homologous RIA system, it was found to be more effective in detecting circulating PAG molecules [9,24]. The average maximum binding rate obtained was in agreement with previously reported specific binding values [9,15,24] and the non specific binding (NSB) value does not exceed the RIA limit of 5% [31]. The minimum detection limit value obtained by the developed direct RIA system was better (<0.8 ng/mL), than those determined with non-preincubated heterologous systems and standard curves ranged from 0.8 to 100 ng/mL [29] and it was in the range (0.3-0.7 ng/mL), of those determinated under the same conditions previously reported [24]. However, with the preincubated heterologous and homologous RIA systems and standard curves ranging from 0.2 to 25 ng/mL, except for the MDL value obtained by Barbato et al.[23], with RIA-706 (0.8 ng/mL), our obtained RIA sensitivity was lower (>0.4 ng/mL) than that previously reported [2,15,22,25,28-30]. The sensitivity of a test is related to the slope of the standard dose-response curve [31] and the use of non-equilibrium methods, which consist of mixing the antiserum and the sample, then after a predetermined time the tracer, has been found useful in some cases to increase the sensitivity of the assay [32]. The difference in the MDL values obtained both in the present study and in the previous ones [2,15,22-25,28-30] can be attributed to the assay procedure, to the solid-phase separation system used, or the calculation of the detection limit, which corresponds to the binding value of duplicate Bo minus 2SD(Bo) for the differents authors. Ayad et al.[33] attributed the differences in RIA assays sensibility to technical aspects, such as the iodination method, the dilution titres of the first antiserum or the incubation time after the addition of the second antibody precipitation system. Physiologically, the detection limit recorded with the developed RIA/PAGs is too close the threshold of 0.8 ng/mL, considered as the basic concentration in PAG assay (RIA-706) for the diagnosis of pregnancy in cows [24], 1.0 ng/mL for ewes [23], goats [2] and for buffalo cows [34]. The wide range concentrations that can be detected by the developed RIA system is sufficient to monitor PAG levels during the gestation of cows [24-29]. Additionally, the system may be very suitable for the determination of PAGs in sheep and goats sera [22,23]. Otherwise, according to Ciabattoni [35], the sensitivity of the assay can be improved by increasing the specific activity of the tracer, thanks to increase in the dose of radiolabeling activity to 1-2 mCi. The results of the analytical validation showed that the non preincubated heterologous RIA system developed (RIA-706) was precise and the accuracy very satisfactory. The performances recorded were comparable to those previously obtained with cows [5,15,33], with sheep [2], with goats [22], with zebu cattle [29] and with buffalo cows [23,30]. Clinically, the measurement of bPAG concentrations in serum samples from cows showed that the RIA-706 developed sytem was able to distinguish non-pregnant cows from pregnant cows and differentiate between each pregnancy stages. it also made it possible, in pregnant cow, after veterinary examination, to detect two cases of fetal mortalities. The PAG values obtained, according to the physiological status and the month of pregnancy of the sampled cows, are in agreement with those reported in previous studies [15,28].

A direct RIA assay to quantify PAGs in bovin sera was developed. The preparation and optimization steps of differents compoments of the kit were presented. Compared to previously reported RIA/PAGs [2,15,22-25,28-30,33], the separation system used was solid phase, requiring only one antibody, one incubation and non centrifugation. The resulting RIA assay was sensitive, reproductible, accurate and was able to distinguish among the serum samples analyzed, between the non-pregnant cows and pregnant ones and within the batch of pregnant cows, to detect cases of fetal mortality. In perspective, this study will be reinforced by another clinical validation, which will include a larger number of cows. the results obtained will be compared with those determined by commercial kit. it would also be wise to include in the study the synthesis of PAGs and the production of specific antibodies.

# Declarations

**Availability of Data and Materials:** The datasets during and/or analyzed during the current study available from the corresponding

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**Ethical Approval:** All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

**Competing Interests:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Author Contribution:** AK, SB, AAA, RK: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. AK, RK, NM: Investigation, Writing - Review & Editing.

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