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Using commercial enzyme immunoassay for measuring pregnancy-associated glycoproteins to diagnose pregnancy in dairy cows under field conditions in Algeria

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Abstract. The purpose of the present work was to study effectiveness for early pregnancy diagnosis in cattle of the new enzyme immunoassay (EIA) sandwich kit commercially available based on the measurement of pregnancy-associated glycoproteins (PAGs). 120 Holstein-Friesian cattle of mixed age and parity were comprised from different dairy herds. The pregnant females (n = 68) were diagnosed by ultrasonography at day 35—40 after artificial insemination and confirmed by transrectal exploration at 2—3 months after AI. The non-pregnant females (n = 52) were housed in the absence of males during the experimental period. Blood samples were collected from coccygeal vessels of females into EDTA tubes. The serum was obtained by centrifugation and the serum was stored at — 20 °C until assay. The PAG concentrations in pregnant and non-pregnant females were determined in serum by EIA kit. The reproducibility inter- and intra-assay of the PAG-EIA is satisfactory (2.78 and 13.19 %, respectively). The accuracy (≥ 94.8 %) and the test of parallelism were largely acceptable. No cross-reaction was observed with the different hormones tested at different dilutions. PAG-EIA system gave 100 % sensitivity and negative predictive values. Whereas, specificity and positive predictive value were 91.93 and 71.15 %, respectively. The accuracy of pregnancy diagnosis by PAG-EIA was 87.5 %. In conclusion, the present study shows clearly that the EIA kit can be used to measure PAG in serum cows for the detection of gestation in Algeria. Therefore, this alternative technique could be recommended to replace the radioactive methods in immunoassays to improve the reproductive performances and an efficient tool for reproductive management of dairy cattle.

Keywords: Pregnancy-associated glycoproteins, enzyme-immunoassay, pregnancy diagnosis, cows

Conflict of interest. The authors declare that they have no conflict of interests

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Introduction

Reproductive management in bovine is an important economic component in the success of a dairy farm. Zootechnical performance needs to be assessed in order to achieve good level and positive financial results to become profitable in bovine herd. The reproductive efficiency of dairy cows increases significantly incomes from milk sales and shorter calving intervals in the modern dairy industry worldwide. Early detection of pregnancy is an essential key to achieve the optimal calving-to-conception interval in dairy cattle. Early pregnancy diagnosis facilitates the detection of non-pregnant cows as early as possible after insemination or early embryonic mortality, and allows them to be re-inseminated. Several techniques are being used to diagnose pregnancy in dairy herd such as transrectal palpation or ultrasonographic examination [1—3].

In a number of mammals, it has been shown that trophoblast cells express a range of pregnancy-associated glycoproteins (PAGs) during pregnancy period. Pregnancy-associated glycoproteins, also known under a variety of other names including pregnancy-specific protein B (PSPB) [4] and pregnancy specific protein 60 (PSP-60) [5], were first described as placental antigens that were present in blood serum of the mother soon after implantation. These proteins appear to be enzymatically inactive as member of the aspartic proteinase family, which also includes pepsin, rennin, cathepsin and several other proteinases [6]. They are synthesized by the mono- and binucleate of the placenta cells, some of them being secreted in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and formation of placentomes begins [7].

Due to the release of secretory granules PAGs in the maternal circulation, it can be used as an indicator of pregnancy and for following up ongoing pregnancies in physiological [8—10] and pathological conditions, such as embryonic and fetal mortalities [5, 11, 12]. These proteins differed in amino acid sequence, molecular masses [13] and degree of glycosylation [14]. Because of large variety of expressed molecules and to large variations in the post translational processing of the glycoproteins, several immunoassay methods, such as radioimmunoassay (RIA) and enzyme immunoassay (EIA), have been developed to be a useful tool of early pregnancy detection in maternal serum [15—18] or milk [19] cattle.

Traditionally, radio-immunoassay (RIA) has been employed to quantify the PAG concentrations in serum using antibody origins. In Algeria, the radioimmunoassay (RIA) methods are less used for routine analysis because of several limitations inherent to the use of radioactive isotopes and it also generates radioactive waste that causes environmental contamination.

In the context, **the purpose of the present work** was to study effectiveness for early pregnancy diagnosis in cattle of the new enzyme-immunoassay (EIA) sandwich kit commercially available based on the measurement of PAGs.

Materials and methods

This research was approved by the Scientific Council Faculty of the Veterinary High National School (Algiers, Algeria). Concerning the ethical aspects, the experimental procedure was performed *in-vitro* and the blood sampling of females was performed according to good veterinary practice under farm conditions.

Animals and samples. This study was conducted in Algeria from March to August 2011. In this study, 120 Holstein-Friesian cattle were comprised from different dairy herds of Tizi-Ouzou (36°46', 4°25') and Biskra (34°49', 5°43') province, Algeria. The age of the animals ranged between 18 months and 12 years with mixed parity. Body condition scores (BCS) of Holstein-Friesian females were noted according to Edmonson et al. [20]. The BCS of experiment females was between 2.5 to 4.5. Animals were examined by a veterinarian and presented no signs of clinical disease. All pregnant females were artificially inseminated (AI) after 100 days post calving [10]. The pregnant cows (Pregnant group, n = 68) were diagnosed by ultrasonography at day 35—40 post-IA (AGRO SCAN A14, sonde bifréquence 3.5 and 5.0 MHz) and confirmed by transrectal exploration at 2—3 months after AI. The non-pregnant heifers (Control group, n = 52) were housed in the absence of males during the experimental period. Blood samples (5 to 8 ml) were collected from coccygeal vessels of Holstein-Friesian females into EDTA tubes (Sarstedt®, Numbrecht, Germany). The serum was obtained by centrifugation (3000 x g at 15 min) and the serum was stored at –20 °C until assay.

PAG enzyme-linked immunosorbent assay. The PAG concentrations in pregnant and non-pregnant females were determined in serum by enzyme immunoassay (EIA) performed in duplicates. The detecting antibody was rabbit anti-PAG IgG as biotin-conjugate. The test sample used a spectrophotometer reader according to the kit instruction. The enzyme substrate was avidin-horseradish peroxidase (HRP). The standard curve ranged from 0 to 2 ng/ml. A cut-off of 0.8 ng/ml was used to discriminate between pregnant and non-pregnant females.

The basis of the test is the sandwich reaction involving two antibodies raised against PAG: the first one is coated on a 96 micro-plate whereas the second one is conjugated to biotin and detected using avidin-HRP. Each sample was analyzed in duplicate. Briefly, dilution buffer is added just before adding PAG standards and serum samples. Afterwards, it is followed by an overnight incubation at room temperature. Microtiter wells are washed before addition of biotinylated anti-PAG. The washing step is followed by incubation with avidin-HRP for 20 min at 37 °C. The plate is washed and after, the substrate/chromogen solution is added to the wells and incubated for 30 min at room temperature. The addition of the stopping reagent transforms the blue coloration into a yellow compound. Finally, the absorption at 450 nm is measured and the optical density is proportional to the PAG concentration.

Technical validation

Reproducibility. To test the reproducibility of EIA kit, two samples with different PAG concentrations were used. Samples were obtained from cows diagnosed earlier with ultrasonography as pregnant. Reproducibility was determined by calculating the intra

and inter-assay of variation (CV) as follow: [%CV = (SD / mean) ×100]. For intra-assay CV, the first serum was assayed in duplicate seven (07) times within the same assay. Likewise, for inter-assay CV, each serum was assayed in duplicate seven (07) times in consecutive assays.

Specificity. To test the specificity of the EIA assay, six (06) reproductive hormones were used, namely PMSG (*Folligon*® 1000 UI, International Intervet B.V., European Union), GnRH (*Fertagyl*®, International Intervet B.V., European Union), progesterone (Progestérone® Retard Pharlon, BAYER health, 13 street Jean Jaurès 92807 Puteaux-cedex, France), testosterone (*Testoenant*®, Geymonat S.P.A. Via S, Anna, 2-Anagni, Italie), oxytocin (*Oxyto-Kel Synth*®, Kela N.V., St. Lenaartseweg 48, 2320 Hoogstraten, Belgium) and dinoprost (*Enzaprost*® T, Cevaanimal health, 10 avenue of ballastière, 33500 Libourne, France). Each hormone was diluted in buffer solution to obtain the following concentrations 1, 10^{-2}, 10^{-3} and 10^{-4}. After that, each dilution of tested compounds was assayed in duplicate.

Parallelism. Parallelism was assessed by serially diluting pregnant cow sera with high PAG concentration. For that, two samples containing relatively high PAG concentrations were diluted in buffer solution and assessed in duplicate. Thus, parallelism of the EIA was determined by evaluating samples at its initial strength (1/1), and at dilutions of 1/2, 1/4, 1/8 and 1/16.

Pregnancy diagnose analysis. This study was designed to test the accuracy of pregnancy outcomes based on PAG-EIA of blood samples collected between 25—50 days post AI. The following table showed the various categories as possible: Sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) and accuracy were determined as described by Kastelic [21]. The sensitivity (Se) was expressed as the proportion of pregnant females with a positive PAG-ELISA result [true positive / (true positive + false negative)]. The Specificity (Sp) was calculated as the proportion of non-pregnant females with a negative PAG-EIA result [true negative / (true negative + false positive)]. Positive predictive value (PPV) was calculated as the proportion of females testing positive that were truly pregnant [true positive / (true positive + false positive)]. Negative predictive value (NPV) was calculated as the proportion of females testing negative that were truly non-pregnant [true negative / (true negative + false negative)].

The accuracy (Ac) was defined as the proportion of pregnant and non-pregnant females correctly identified by the test [(true positive + true negative) / (true positive + true negative + false positive + false negative)]. The rate of false positive or negative result is the likelihood of a positive or negative result in cows known not to be pregnant or be pregnant, and this rate is related to the test specificity [rate of false positive = 1 — specificity] or sensitivity [rate of false negative = 1 — sensitivity], respectively [22].

Statistical analysis. Statistical analyses were carried out in STATVIEW (Version 4.55). Statistical analysis was performed using t-test to compare treated and control females. The PAG concentrations were expressed as mean ± SD, and $P < 0.05$ was considered significant. The statistical significance of test was determined by Student tests.

Results

The standard curves (n = 6) used ranged from 0.4 to 2 ng/ml to estimate PAG concentrations with a linear regression plot (Fig. 1). The reproducibility of PAG-EIA technical are summarized in Table 1. The values of inter-assay CV are 6.08 and 13.19 %, whereas intra-assay CV is low calculated for PAG-EIA (2.78 %). The recovery rates obtained by the use of PAG-EIA technical were higher 96.8 %, as shown in Table 2. Parallelism of serum samples diluted with buffer solution is given in Table 3. As far as the specificity is considered, no cross reaction of PAG-EIA was recorded for reproduction hormones tested at different dilutions (1, 10^{-2}, 10^{-3} and 10^{-4} IU/ml).

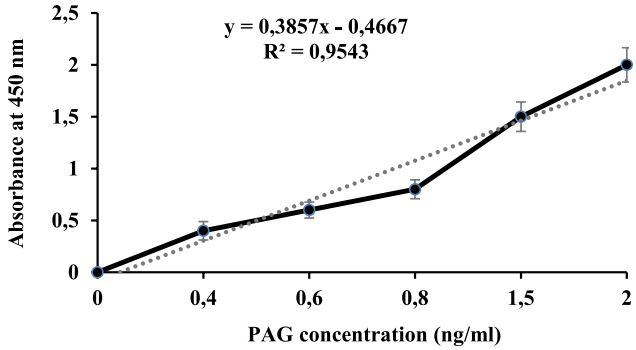

Fig. 1. Standard curves (mean ± SD) of pregnancy-associated glycoproteins (PAG) in the enzyme immunoassay calculated over six assays

Table 1

Intra- and inter-assay coefficients of variation of pregnancy-associated glycoproteins by ELIA

Sample	Intra-assay[a]		Inter-assay[b]	
	PAG concentration[c], ng/ml	CV, %	PAG concentration[c], ng/ml	CV, %
1	2.21± 0.06	2.78	2.33 ± 0.14	6.08
2	–	–	0.94 ± 0.12	13.19

[a]Seven replicates in the same assay. [b]Two replicates in 7 consecutive assays. [c]Mean ± SD.

Table 2

Recovery of different concentrations of PAG added to a plasma sample containing low concentrations of PAG respectively as measured by ELIA

Theatrical PAG concentration, ng/ml	Observed PAG concentration, ng/ml	Recovery[a], %
1.25	1.21	96.8
1.20	1.14	95
1.16	1.10	94.8

[a](Observed value/Theatrical value) × 100.

Table 3

Serial dilutions of a plasma sample containing relatively high PAG concentrations as measured by EIA

Dilution[a]	PAG concentration, ng/ml	
	Sample 1	Sample 2
1/1	5.16	5.18
1/2	3.53	3.61
1/4	2.21	2.07
1/8	1.30	1.17
1/16	0.65	–

[a]Samples from pregnant females were diluted in buffer solution.

The PAG concentration (mean ± SD) determined in serum samples from non-pregnant and pregnant females is represented in Table 4. PAG concentrations measured by EIA system are significantly (P < 0.001) higher in pregnant females than in non-pregnant females (3.51 ± 1.17 vs. 0.53 ± 0.08). It can be seen that in pregnant cows, the PAG-EIA resulted in the high and the least variable concentrations than the threshold currently used for pregnancy diagnosis (0.80 ng/mL). For samples of non-pregnant females, all PAG concentrations revealed very low content than the threshold 0.80 ng/mL.

Table 4

Mean (± SD) pregnancy-associated glycoprotein concentrations obtained by EIA in pregnant and non-pregnant female

	Non-pregnant females (*n* = 52)	Pregnant females (*n* = 68)
PAG concentration (mean± SD, ng/ml)	0.53 ± 0.08[a]	3.51 ± 1.17[b]
Min-Max	0.06—0.78	0.98—6.21

[a, b]Significant differences between PAG concentrations from pregnant and non-pregnant females ($P < 0.001$).

The sensitive, specificity, positive and negative predictive values of the PAG-EIA tests are presented in Table 5. PAG-EIA system gave 100 % sensitivity and negative predictive values. Whereas, specificity and positive predictive value were 91.93 and 71.15 %, respectively. From a total of 52 samples collected in non-pregnant cows, measurement by PAG-EIA technique resulted in 15 cases of incorrect pregnancy diagnosis, i.e. false positive. The accuracy of pregnancy diagnosis by PAG-EIA was 87.5 %. There was a significant increase of PAG concentration from Day 25 to Day 50 after AI measured by EIA system ($P < 0.05$) (Fig. 2). Positive correlation between PAG concentrations AI measured by EIA and day after AI was r = 0.32 ($P \leq 0.001$).

Table 5

Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of PAG-EIA for determining pregnancy status 25—50 days post AI

Variables	PAG-EIA
Correct positive results, *n*	68
False positives, *n*	15
Correct negative results, *n*	37
False negatives, *n*	00
Sensitivity Se, %	100
Specificity Sp, %	91.93
Positive predictive value PPV, %	71.15
Negative predictive value NPV, %	100
Accuracy Ac, %	87.5
Rate of false positive, %	8.07
Rate of false negative, %	0

Se = true positive / (true positive + false negative). Sp = true negative / (true negative + false positive). PPV = true positive / (true positive + false positive). NPV = true negative / (true negative + false negative). Ac = (true positive + true negative) / (true positive + true negative + false positive + false negative). Rate of false positive = 1 — specificity. Rate of false negative = 1 — sensitivity.

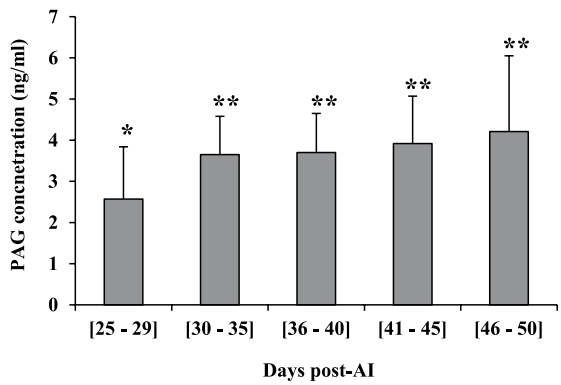

Fig. 2. Plasma concentrations of PAG, ng/ml, measured by enzyme immunoassay system during the first trimester of pregnancy (25…50 post AI) in dairy cows: *,** — values with different superscripts of PAG concentration differ statistically ($P < 0.05$)

Discussion

The gestation diagnosis is of crucial importance to the farmer for the establishment and maintaining the production performances and dairy herd reproductive management. Early detection of pregnancy plays a key role in the achievement of an optimal

calving-to-conception interval in dairy and beef cattle. For this, it requires effective diagnostic methods that are accurate, practical. The results of this investigation have demonstrated that using a commercial EIA test to detect serum PAG concentrations was a highly accurate form of early pregnancy diagnosis in cattle under field conditions in Northern Algeria.

The test of reproducibility inter- and intra assay evaluated with bovine serum of the PAG-EIA system is satisfactory. CV inter- and intra-assay of PAG as measured by ELISA were reported by Gatea and co-authors [23] to be 7.8 and 8 %, respectively. These slight differences can be attributed to materials used and user experience. Parallelism was assessed by a serial dilutions (1/1, 1/2, 1/4 and 1/8) with pregnant female containing a high PAG concentration. In the present study, the results obtained show clearly that the concentrations PAG obtained are parallel. Concerning the precision of PAG-EIA, the results obtained are acceptable with the rate of recovery 94.8 %. The specificity test carried out here allowed us to verify the hypothetical interference of reproductive hormones and other placental glycoproteins on commercial PAG-EIA system. Our results showed that the commercial EIA was specific for the detection of PAG with regard to PMSG, GnRH, progesterone, testosterone, oxytocin and Prostaglandin F2α. It is common knowledge that some placental glycoproteins from human and equine origin presented probably similar to those observed in the PAG molecules [24, 25].

The accuracy of pregnancy diagnosis is important criteria in relation to sensitivity and specificity values. In the current study, all pregnant females were correctly diagnosed as pregnant by PAG-EIA, while the low rate of non-pregnant females was diagnosed incorrectly. The sensitivity and the specificity of the commercial PAG-EIA at Day 28—50 after AI were high (100 and 91.93 %, respectively) for using as tool of pregnancy diagnosis in cattle. This result allows to reduce false-negative diagnosis, and the risk of prostaglandin injection in pregnant females in cattle breeding. Thus, it is desirable for the sensitivity to be very high (100 %) [26] because this can reduce the calving interval by re-inseminating of non-pregnant cows as early as possible. The sensitivity and negative predictive value outcomes of our study is similar to that reported by numerous authors using RIA-PAG [10, 15, 27], and using PAG- or PSPB-ELISA [26, 28, 29]. Recently, Meziane et al. [30] compared and evaluated two methods of pregnancy diagnosis, namely proteins associated with pregnancy and ultrasonography, in dairy cattle in the East of Algeria. The sensitivity and the specificity of PAG by the ELISA test were similar (100 and 93.75 %, respectively) to our results. Likewise, the sensitivity rate reported in the present study was higher than those from the previous findings [18, 31, 32]. The specificity of the PAG-EIA test for diagnosing non-pregnant cows obtained in the present study was higher than those reported, ranging from 66 to 90.7 %, in the researches assessing the effectiveness of the PAG-EIA test at Days 26—30 [16, 32—35]. Also, other investigations reported the specificity similar or slightly higher (91.1—97.2 %) carried out with the PAG-EIA test [18, 29, 31, 36] and the PAG-RIA [15, 27, 29] in dairy cows in comparison to the present study. These variations could be due to the interference in the assay of sample constituents rather than to the hormone to be measured. The discrepancy in the technique accuracy to predict pregnancy might be explained by the specific PAGs being detected by antibodies employed in the PAG-EIA. It is important to remember that

the molecular biology researches have been estimated over 100 PAG genes in ruminant genome most of them being expressed in the superficial layers of the placenta [37]. Recently, Ayad and Touati [38] concluded there was another source of glycoprotein expression apart from the placenta in cow. A similar finding of the detection of PAG in testicular tissue and an ovarian extract has also been demonstrated by Zoli et al. [39].

In our work, the increased PAG concentration in the first trimester pregnancy was similar to previous researches [40—42]. These variation of PAG concentration in dairy cows may be affected by the nutritional status and body score. Lopez-Gatius et al. [43] reported lower PAG concentrations in high producing dairy cows, which can partially explain differences between PAG concentrations found in dairy cattle in our study. Also, these divergence of serum PAG levels after AI might be attributed to the parity [41].

Conclusion

The present study shows clearly that the enzyme immunoassay kit can be used to measure PAG in serum of cows for the detection of gestation in Algeria. It is well known that the RIA method presents limitations to the use of radioactive isotopes in several countries, especially in Africa, because of concerns for radiation safety, short shelf lives of radioactive reagents and radioactive waste disposal. Therefore, this alternative technique could be recommended to replace the radioactive methods in immunoassays to improve the reproductive performances and an efficient tool for reproductive management of dairy cattle.

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Использование иммуноферментного анализа для определения гликопротеинов беременности с целью диагностики стельности дойных коров в полевых условиях Алжира

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Аннотация. Изучена эффективность ранней диагностики беременности у крупного рогатого скота в полевых условиях в Алжире с помощью нового доступного набора для иммуноферментного анализа, основанного на определении гликопротеинов, связанных с беременностью. Исследованы 120 коров голштино-фризской породы смешанного возраста из разных молочных стад. Контроль беременности у 68 стельных коров осуществляли методом ультразвуковой диагностики на 35…40-й день после инсеминации и подтверждали трансректальным исследованием через 2—3 месяца после проведения искусственного осеменения. 52 нестельных коров содержали изолированно от самцов на протяжении всего эксперимента. Образцы крови отбирались из хвостовой артерии в пробирки с ЭДТА. Плазму получали центрифугированием и хранили при температуре –20 °C до начала проведения анализа. Концентрации гликопротеинов беременности (ГПБ) у стельных и нестельных коров определяли в плазме крови с помощью набора реагентов ИФА. Воспроизводимость результатов ГПБ-ИФА (внутри- и межсерийной сходимости) удовлетворительна (2,78 и 13,19 % соответственно). Точность (≥ 94,8 %) и повторяемость анализа были в основном приемлемыми. Не наблюдалось перекрестной реакции с разными гормонами, протестированными в разных разведениях. Диагностическая чувствительность ГПБ-ИФА тест-системы составила 100 % и наблюдалась отрицательная прогностическая значимость. При этом специфичность и прогностическая ценность положительного результата составили 91,93 и 71,15 % соответственно. Точность диагностики беременности с помощью ГПБ-ИФА составила 87,5 %. Таким образом, настоящее исследование явно показывает, что набор для ИФА можно использовать для измерения содержания ГПБ в сыворотке крови коров с целью определения стельности. Следовательно, данный альтернативный метод может быть рекомендован для замены методов иммуноанализа с применением радиоактивных изотопов для улучшения репродуктивных показателей коров и является эффективным инструментом для воспроизводства молочного крупного рогатого скота.

Ключевые слова: гликопротеины беременности, иммуноферментный анализ, диагностика беременности, коровы

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