Original article

Detection of pregnancy by radioimmunoassay of a pregnancy serum protein (PSP60) in cattle

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Summary — The accuracy and efficiency of pregnancy diagnoses in cattle by pregnancy serum protein (PSP60) radioimmunoassay, a progesterone radioimmunoassay or oestrus detection were compared. Blood samples were taken from 349 suckling heifers and cows (1 191 inseminations) at 28, 35, 50 and 90 d post-insemination for PSP60 determination and at 22–23 d for progesterone. Females were declared nonpregnant when plasma PSP60 concentration was lower than 0.2 ng/ml at 28, 35 and 50 d and 0.5 ng/ml at 90 d. When compared with rectal palpation at 90 d, the accuracy of positive (negative) diagnoses by progesterone assay was 80% (100%) in heifers and 75% (99%) in cows. The accuracy of positive diagnoses by PSP60 assay increased with gestation stage from 90% on d 28 in heifers (74% in cows) to 100% (99% in cows) at the time of rectal palpation. This accuracy was 84% on d 28 in cows when the interval from calving to blood sampling was higher than 115 d. Whatever the stage, the accuracy of negative diagnoses was higher than 90%. Efficiency in detecting pregnant or nonpregnant females on d 28 was equivalent to the progesterone assay. The method for detecting oestrus applied in this experiment was as efficient as the PSP60 or progesterone test at any stage of gestation. The PSP60 test is very flexible, which makes its use particularly interesting in naturally mated suckling herds because of the uncertainty regarding the date of fertilization.

pregnancy diagnosis / pregnancy protein / progesterone / oestrus detection / radioimmunoassay / beef cattle

Résumé — Diagnostic de gestation chez les bovins par dosage radio-immunologique d'une protéine sérique de gestation (PSP60). *Nous avons comparé l'exactitude et la fiabilité de diagnostics de gestation chez les bovins par dosage radio-immunologique d'une protéine sérique de gestation (PSP60) et de la progestérone et par détection des chaleurs. Les prélèvements sanguins ont été effectués sur 349 génisses et vaches allaitantes (1 191 inséminations) aux stades 28, 35, 50 et 90 j post-insémination pour doser la PSP60 et à 22–23 j pour la progestérone. Les femelles ont été déclarées non gravides pour une concentration plasmatique de PSP60 inférieure à 0,2 ng/ml aux 3 premiers stades et 0,5 ng/ml à 90 j. Par comparaison à la palpation rectale à 90 j, l'exactitude des diagnostics positifs (respectivement négatifs) par dosage de progestérone est de 80% (respectivement 100%) chez les génisses et 75% (respectivement 99%) chez les vaches. L'exactitude des diagnostics positifs par dosage de PSP60 augmente avec le stade de gestation de 90% à 28 j chez les génisses (respectivement 74% chez les vaches) à 100% (respectivement 99%) au moment du palper rectal. Chez les vaches, quand l'intervalle vêlage–prise de sang est supérieur à 115 j cette exactitude est de 84% à 28 j. Quel que soit le stade de gestation, l'exactitude des diagnostics négatifs est*

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supérieure à 90%. La fiabilité de détection des femelles gravides ou non gravides à 28 j équivaut à celle de la progestérone. La méthode de détection des chaleurs appliquée dans cette expérience présente la même efficacité que les dosages de PSP60 ou de progestérone, à stade équivalent. Le test PSP60 présente une grande souplesse d'utilisation, ce qui le rend particulièrement intéressant en troupeaux allaitants conduits en monte naturelle, du fait de l'incertitude de la date de fécondation.

diagnostic de gestation / protéine de gestation / progestérone / détection des chaleurs / dosages radio-immunologiques / bovins à viande

INTRODUCTION

Pregnancy in cattle can be detected by using different types of methods: observation of oestrus; methods based on physical changes in the uterus of the pregnant cow; and methods based on biochemical modifications in blood or milk. The first method is traditionally used in the field. Observation of physical changes in the uterus can be carried out using ultrasonic detection or rectal palpation. To be really efficient, these 2 methods require regular practice by the technician or the veterinary and should not be used before the 35–40th d of pregnancy. The progesterone assay in milk or blood, around 21 d after artificial insemination (AI) in the cow, is the earliest diagnosis presently available. However, this test is not specific to pregnancy because progesterone is also secreted during the luteal phase of the oestrous cycle. This test also requires that the fertilization date be precisely known. Females in suckling herds are generally bred by natural mating, in which case pregnancy diagnosis by the progesterone assay is of little use because the mating date is unknown.

In the search for a specific method for detecting pregnancy, it seemed interesting to assay proteins secreted by the placenta and detectable in maternal circulation, as has been performed for many years in humans using the hCG assay (Marshall *et al,* 1968).

Specific markers have already been assayed in order to detect pregnancy in cattle. PSPB (pregnancy specific protein B) a family of 5 glycoproteins secreted by the trophoblast, has been characterized (Butler *et al,* 1982; Crock *et al,* 1988). The radioimmunoassay (RIA) of PSPB has been successfully developed as a pregnancy diagnosis suitable 30 d after AI and beyond 70 d *post partum* in dairy cows (Humblot *et al,* 1988a). Recently, Zoli *et al* (1991) have purified and characterized a bovine pregnancy-associated glycoprotein (bPAG). These authors performed pregnancy diagnoses in cows from 30 d post-conception onwards by detecting this placental-specific antigen in the serum (Zoli *et al,* 1992).

A pregnancy serum protein, with a molecular weight of 60 kD (PSP60) has also been isolated and purified from bovine foetal cotyledons at 168 d of pregnancy (Camous *et al,* 1988). The profile of secretion was described in a previous study (Mialon *et al,* 1993). By comparing the amino-acid sequences of PSP60 (INRA patent No FR 88 03590 filed March 18, 1988), bPAG (Xie *et al,* 1991) and PSPB (Lynch *et al,* 1992), it was suggested that the proteins bPAG and PSP60 may correspond to various degrees of glycosylation of 1 of the 5 forms of PSPB. The purpose of the present study was to test this new protein assay as a pregnancy diagnosis in suckling herds. The validation of the PSP60 assay as a pregnancy diagnosis was checked in an experimental suckling herd managed by AI.

MATERIALS AND METHODS

Animals

This study dealt with a suckling herd of 349 Charolais heifers or cows kept in free stall areas on an experimental INRA farm. These females were inseminated several times during the 1987–1990 period and a total of 1 191 inseminations were studied.

Experimental study of pregnancy

A vasectomized bull was introduced in the herd twice a day for detecting which females were in oestrus. Females were inseminated after each observed oestrus, and pregnancy was studied using different methods as long as the female was not observed in oestrus again. These methods were: i) early pregnancy diagnosis performed by progesterone RIA from plasma samples 21.8 ± 1.1 d after AI; ii) diagnoses based on PSP60 concentrations. Taking into account the PSP60 secretion profile in early pregnancy (Mialon et al, 1993), the first sample was taken at 27.5 ± 0.6 d (d 28). Three further samples were taken at 33.7 ± 1.1 d (d 35), 49.5 ± 3.1 d (d 50), and 89.9 ± 4.4 d (d 90); and iii) rectal palpation at 89.9 ± 3.5 d post-AI to confirm pregnancy in females not observed in oestrus.

Blood sampling and assay procedures

A 5-ml blood sample was collected from the caudal vein into heparinized vacutainers for each assay. Plasma was separated by centrifuging within 1 h of sampling and stored at $-20°C$ until assayed.

Progesterone concentrations were assayed on 100-μl samples of plasma with a rabbit antiserum used at a final dilution of 1:25 000 according to an RIA procedure previously described by Terqui and Thimonier (1974). An early pregnancy diagnosis was then performed as described in Thimonier (1973).

Plasma concentrations of PSP60 were measured with an RIA developed by Camous et al (INRA patent No FR 88 03590 filed March 18, 1988). PSP60 assays were performed on four 100-μl aliquots of pure or diluted (with plasma from a nonpregnant cow) plasma. All blood samples from the same female were tested simultaneously. Rabbit antiserum against PSP60 was used at a final dilution of 1:1 800 000. There was no cross-reactivity with either ovine luteinizing hormone (oLH) or ovine follicle stimulating hormone (oFSH). Cross-reactivity with ovine placental lactogen (oPL) was minimal (< 0.003%). Sensitivity of the assay was 0.2 ng/ml plasma. Intra- and inter-assay coefficients of variation were 6 and 12%, respectively, when estimated with a reference plasma of 1.3 ng/ml.

Determination of diagnosis results

Cows and heifers were declared pregnant by PSP60 assay when the plasma PSP60 concentration was higher than 0.2 ng/ml at d 28, 35, 50 and higher than 0.5 ng/ml at d 90. A PSP60 concentration between 0.2 and 0.5 ng/ml at 90 d after AI actually proves that a pregnancy has been interrupted (Mialon et al, 1993). For oestrus detection, a female which was not observed in oestrus until d 22 (or 28, 35, 50, 90) was declared pregnant at d 22 (or 28, 35, 50, 90, respectively). The pregnancy test was declared negative if a female was observed in oestrus, and positive otherwise. The accuracy and the efficiency of the pregnancy diagnosis were obtained by comparing results of each test with results of rectal palpation at 90 d after AI. The rate of late abortion, ie after 90 d, is generally low. Late abortion occurred in 2% of the AIs performed in this experiment.

These diagnosis results were used to compute various accuracy and efficiency ratios.

Accuracy

The accuracy of a pregnancy diagnosis is the proportion of (positive or negative) diagnoses confirmed by rectal palpation at 90 d among those (positive or negative) performed by either progesterone or PSP60 assays.

The accuracy of positive diagnosis was:

$$= \frac{\text{N° confirmed positive diagnoses} \times 100}{\text{N° positive diagnoses}}$$

The accuracy of negative diagnosis was:

$$= \frac{\text{N° confirmed negative diagnoses} \times 100}{\text{N° negative diagnoses}}$$

Efficiency

The efficiency of the positive (or negative) diagnoses is the proportion of pregnant (or nonpregnant) females actually detected by the pregnancy diagnosis.

The efficiency of positive diagnoses was:

$$= \frac{\text{N° confirmed positive diagnoses} \times 100}{\text{N° pregnant females at 90 d}}$$

The efficiency of negative diagnoses was:

$$= \frac{\text{N° confirmed negative diagnoses} \times 100}{\text{N° nonpregnant females at 90 d}}$$

The accuracy and efficiency of the different techniques at different sampling stages for detecting pregnant and nonpregnant females were subsequently compared by Chi-squared tests or using hypergeometric distributions when needed.

RESULTS

After 4 reproduction seasons in the suckling Charolais herd, 303 heifers and 347 cows were confirmed pregnant at 90 d by rectal palpation out of a total number of 1 191 AIs.
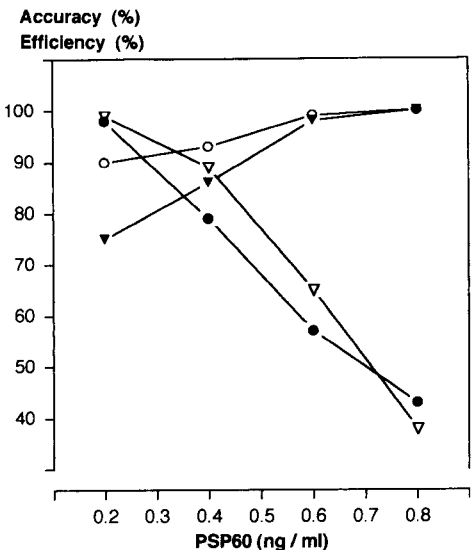
Accuracy of the pregnancy diagnosis by PSP60 assay

In heifers as in cows, the accuracy of positive results by the PSP60 assay increased with pregnancy stage (table I). The accuracy of positive diagnoses was higher in heifers than in cows up to and including the d-

50 stage. In cows, we studied the accuracy of positive pregnancy diagnoses according to the interval from calving to blood sampling since the PSP60 remains detectable in blood a few weeks after parturition (Mialon *et al*, 1993). Thus, we may note (table II) that the further the diagnosis is from the previous calving, the lower the proportion of inaccurate positive results.

The accuracy of negative PSP60 results was higher than 90% as early as 28 d in both heifers and cows (table I). The few inaccurate negative PSP60 results were due to females with a late secretion of PSP60, which began later than 50 d of pregnancy in some cases.

Changing the threshold of PSP60 concentration at which females were declared pregnant from 0.2 to 0.6 ng/ml increased the accuracy of positive results at d 28 from 90 to 99% in heifers whereas it decreased that of negative results (fig 1).

Fig 1. Accuracy and efficiency of pregnancy diagnosis by PSP60 RIA at d 28 in suckling heifers according to an increasing threshold of PSP60 concentration: O: positive accuracy; ●: negative accuracy; ∇: positive efficiency; ▼: negative efficiency.

Table I. Accuracy of pregnancy diagnosis by progesterone RIA at d 22 post-AI, PSP60 RIA at d 28, 35, 50 and 90, and oestrus detection in a suckling Charolais herd.

Pregnancy diagnosis	Accuracy							
	Positive				Negative			
	Oestrus		Assay		Oestrus		Assay	
	%*	N	%*	N	%*	N	%*	N
Day 22								
Heifers	85[cd]	302/357	80[de]	155/194	99[a]	139/140	100[a]	63/63
Cows	68[f]	343/504	75[e]	292/388	98[a]	186/190	99[a]	188/190
Day 28								
Heifers	87[c]	302/346	90[bc]	120/134	99[a]	150/151	98[ab]	43/44
Cows	74[e]	343/463	74[ef]	211/286	98[a]	227/231	92[b]	109/119
Day 35								
Heifers	90[bc]	301/336	92[bc]	281/307	99[a]	159/161	94[ab]	32/34
Cows	77[e]	341/444	78[e]	318/409	98[ab]	244/250	99[a]	147/149
Day 50								
Heifers	93[b]	301/323	94[b]	243/258	99[a]	172/174	93[ab]	14/15
Cows	87[c]	340/391	87[c]	336/388	98[ab]	296/303	96[ab]	75/78
Day 90								
Heifers	96[b]	300/311	100[a]	300/301	98[a]	183/186	96[ab]	23/24
Cows	92[b]	336/364	99[a]	333/336	97[a]	319/330	99[a]	89/90

* Means with different superscripts differ significantly ($P < 0.05$).

Compared accuracies of pregnancy diagnoses by PSP60 and progesterone RIA and by oestrus detection

As early as 28 d of pregnancy, the accuracy of positive PSP60 diagnoses in heifers was significantly higher than the accuracy of positive progesterone results at 22 d (table I). The same was observed with cows provided the d 28 sample was collected at least 115 d after the previous calving (table II). Positive diagnoses by PSP60 RIA were significantly more accurate in all cows than those obtained with the progesterone assay (from 50 d post-AI onwards only) what-ever the interval between calving and blood sampling (table I). The accuracy of negative PSP60 results was greater than 90% and generally equivalent to that of the progesterone assay whatever the stage of pregnancy and the age of females. The accuracy of positive results of oestrus detection appeared to be equivalent to the pregnancy diagnoses by progesterone (d 22) or PSP60 (d 28 and after) assays at the same stages, except for cows at d 22 and heifers and cows at d 90. At d 22, positive diagnosis by progesterone assay was significantly more accurate than the corresponding oestrus observation in cows. At d

Table II. Accuracy of positive pregnancy diagnosis by PSP60 RIA 28 d after AI according to interval between calving and blood sampling.

Interval between calving and blood sampling (d)	%	N
≥ 85	75[a]	205/274
≥ 95	75[a]	195/260
≥ 105	78[ab]	181/231
≥ 115	84[b]	158/189
Total	74[a]	211/286

[ab] Means with different superscripts differ significantly ($P < 0.05$).

90, the positive diagnosis by PSP60 RIA was significantly more accurate than oestrus detection in both heifers and cows. The accuracy of negative results based on oestrus observation was generally equivalent to those based on an RIA.

Compared efficiencies of PSP60 or progesterone RIA and observation of oestrus as pregnancy (or nonpregnancy) tests

As early as 28 d, the PSP60 assay enabled 95 and 99% of pregnant cows and heifers, respectively, to be detected. These results were close to the efficiency of the progesterone assay: 99 and 100%, for cows and heifers respectively (table III). The efficiency of positive pregnancy diagnoses based on a lack of oestrus before d 22 or d 28 (99 and 100% for cows and heifers, respectively) did not differ from the efficiencies of PSP60 and progesterone detection (table III).

Detection of nonpregnant females was much less efficient whatever the method used. Only 54 to 77% of the females that were not pregnant on d 90 could actually be detected as early as d 22 or d 28. The results were generally better with heifers (62 to 77%) than with cows (54 to 66%) and the 3 methods did not appear to differ

Table III. Detection efficiency of pregnant and nonpregnant females by progesterone RIA at d 22, PSP60 RIA at d 28 and oestrus detection at d 22 and 28 post-AI.

Pregnancy diagnosis	Efficiency							
	Positive				Negative			
	Oestrus		Assay		Oestrus		Assay	
	%*	N	%*	N	%*	N	%*	N
Day 22								
Heifers	100[a]	302/303	100[a]	155/155	72[ab]	139/194	62[bc]	63/102
Cows	99[a]	343/347	99[a]	292/294	54[c]	186/347	66[bc]	188/284
Day 28								
Heifers	100[a]	302/303	99[a]	120/121	77[a]	150/194	75[ab]	43/57
Cows	99[a]	343/347	95[a]	211/221	65[bc]	225/347	59[c]	109/184

* Means with different superscripts differ significantly ($P < 0.05$); * using the hypergeometric distribution.

significantly. Moreover, setting the PSP60 concentration threshold at 0.6 ng/ml at d 28 instead of 0.2 ng/ml increased the efficiency of negative PSP60 diagnoses from 75 to 98% (fig 1).

DISCUSSION

The pregnancy diagnosis based on the PSP60 RIA is pregnancy specific because it allows the detection of a conceptus-secreted protein. The accuracy of positive pregnancy diagnoses shows that the PSP60 RIA at d 28 is a better pregnancy test than the progesterone assay in heifers (90 vs 80%) and in cows if sampled at least 115 d post partum (84 vs 75%). The PSP60 test at day 28 detects pregnant and non-pregnant females as efficiently as the progesterone assay at d 22 and as oestrus detection up to d 22 or d 28. Embryonic and foetal mortalities that occur between diagnosis and palpation at 90 d explain why the females that were not pregnant (at 90 d) could not all be detected early. The efficiency of negative PSP60 diagnoses can be improved by increasing the concentration threshold used to decide whether a female is pregnant or not since it has been shown that late embryonic mortality tends to be associated with a lower PSP60 concentration at 28 or 35 d (Mialon et al, 1993).

The PSP60 test may only be applied from 27 d after AI, for all females. It was shown that, from this stage onwards, nearly 100% of the pregnant females had a concentration of PSP60 equivalent to or higher than 0.2 ng/ml, which is detectable by the RIA procedure (Mialon et al, 1993). However, this test is of restricted use in cows after calving. The initial study showed that the blood still contained residual concentrations of PSP60 for 13 weeks after calving in dairy cows, and 15 weeks in suckling cows (Mialon et al, 1993). These residual concentrations may lead to

inaccurate positive diagnoses. The PSP60 diagnosis is only useful for obtaining highly reliable positive diagnoses in cows from 100–110 d after the previous calving. Humblot et al (1988b) also reported that residual concentrations of PSPB were still detectable in maternal blood 100 d after calving in 15% of dairy cows. The accuracy of the positive results obtained by Humblot et al (1988a) with the PSPB RIA in dairy females is similar to those we obtained with PSP60 concentrations in suckling heifers. Detecting pregnancy by RIA for bPAG in dairy heifers with a threshold of 0.5 ng/ml, Zoli et al (1992) obtained an accuracy of 93% at 35 d when pregnancy was confirmed at 45 d by rectal palpation. These results suggest that these 3 tests based on assaying pregnancy proteins are equivalent for detecting pregnancy in cattle.

The PSP60 test has to be performed later than the progesterone diagnosis. Nevertheless, the PSP60 assay, the PSPB assay (Humblot et al, 1988a) and the bPAG assay (Zoli et al, 1992) are more flexible to use, which makes them particularly interesting for pregnancy diagnosis in suckling herds where natural mating is still widely practiced. The date of mating or AI is not needed with these 3 protein assays. The test need only be applied at least 1 month after fertilization. Such a pregnancy diagnosis can thus be carried out in the herd at the end of a joining season to estimate reproductive efficiency. For the breeder, this test is reliable, easier to use than rectal palpation, less expensive than ultrasonic detection, but requires a few days' delay to obtain the result. The PSP60 test appeared as accurate as detecting oestrus using a vasectomized bull twice a day from AI to d 22 or later in our study. However, such a plan of oestrus detection is too heavy and thus not feasible in the field, unlike the PSP60 test. Besides, as shown in the initial study (Mialon et al, 1993), the PSP60 test can be performed several

times on each animal to observe the timing of the pregnancy by examining the kinetics of secretion of the PSP60. This test is therefore also of great interest in experimental farms where cattle reproduction is studied.

In conclusion, the plasma PSP60 RIA appears to be useful for both diagnosing pregnancy and detecting nonpregnant females. Its detection efficiency is maximal as early as 27–30 d after AI for suckling Charolais females mated at least 80 d *post partum*. Although it is a late pregnancy diagnosis, this test is particularly useful for suckling herds.

ACKNOWLEDGMENTS

REFERENCES

Butler JE, Hamilton WC, Sasser RG, Ruder CA, Hass GM, Williams RJ (1982) Detection and partial characterization of two bovine pregnancy-specific proteins. *Biol Reprod* 26, 925-933

Camous S, Charpigny G, Guillomot M, Martal J (1988) Purification of one pregnancy-specific protein by high-performance liquid chromatography (HPLC). *In: Proc Bard Workshop on maternal recognition of pregnancy and maintenancy of the corpus luteum.* Jerusalem, 20-24 Mars, abstr 2

Crock JE, Ruder-Montgomery CA, Sasser RG (1988) Characteristics of bovine pregnancy-specific protein B (bPSPB). *Biol Reprod* 38 (suppl 1), 78 abstr 88

Humblot P, Camous S, Martal J, Charlery J, Jeanguyot N, Thibier M, Sasser RG (1988a) Diagnosis of pregnancy by radioimmunoassay of a pregnancy-specific protein in the plasma of dairy cows. *Theriogenology* 30, 257-267

Humblot P, Jeanguyot N, Ruder CA, Leriche I, Thibier M, Sasser RG (1988b) Accuracy of pregnancy diagnosis by PSPB RIA in the plasma of dairy cows 28 days after AI. *In: Proc 11th Int Congr Anim Reprod and Artif Insem,* Dublin (short communication), vol 2 p 94

Lynch RA, Alexander BM, Sasser RG (1992) The cloning and expression of the bovine pregnancy specific protein B (bPSPB). *Biol Reprod* 46 (suppl 1), 73 abstr 89

Marshall JR, Hammond CB, Ross GT, Jacobson A, Rayford P, Odell WD (1968) Plasma and urinary chorionic gonadotrophin during early human pregnancy. *Obstet Gynecol* 32, 760-764

Mialon MM, Camous S, Renand G, Martal J, Menissier F (1993) Peripheral concentrations of a 60-kD pregnancy serum protein during gestation and after calving and in relationship to embryonic mortality in cattle. *Reprod Nutr Dev* 33, 269-282

Terqui M, Thimonier J (1974) Nouvelle méthode radioimmunologique rapide pour l'estimation du niveau de progesterone plasmatique. Application pour le diagnostic précoce de la gestation chez la brebis et la chèvre. *CR Acad Sci* (Paris), Sér D, 279, 1109-1112

Thimonier J (1973) Diagnostic précoce de la gestation par l'estimation de progestérone plasmatique chez la brebis, la vache et la jument. *Rec Med Vet Ec Alfort* 149, 1303-1318

Xie S, Low BG, Nagel RJ, Kramer KK, Anthony RV, Zoli AP, Beckers JF, Roberts RM (1991) Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. *Proc Natl Acad Sci USA* 88, 10247-10251

Zoli AP, Beckers JF, Wouters-Ballman P, Closset J, Falmagne P, Ectors F (1991) Purification and characterization of a bovine pregnancy-associated glycoprotein. *Biol Reprod* 45, 1-10

Zoli AP, Guilbault LA, Delahaut P, Benitez Ortiz W, Beckers JF (1992) Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. *Biol Reprod* 46, 83-92