J. Dairy Sci. 84:1884–1889

Assessment of a Commercially Available Early Conception Factor (ECF) Test for Determining Pregnancy Status of Dairy Cattle

M. C. Cordoba, R. Sartori, and P. M. Fricke
Department of Dairy Science,
University of Wisconsin, Madison 53706

ABSTRACT

The Early Conception Factor (ECF) test is a commercially available qualitative assay that reportedly detects a pregnancy-associated glycoprotein present in bovine serum within 48 h after conception. One concern with previous assessments of this test is that animals with viable embryos early during pregnancy that subsequently undergo embryonic loss before pregnancy diagnosis increase the rate of false-positive results and bias the assessment. To preclude this possibility, noninseminated Holstein cows (n = 9) and heifers (n = 8) were evaluated as an unequivocal source of nonpregnant animals, and Holstein cows (n = 17) and heifers (n = 1) inseminated at estrus and in which at least one embryo of transferable quality was recovered at a nonsurgical flush 6 d after artificial insemination were evaluated as an unequivocal source of pregnant animals. Blood samples were collected from all animals 6 d after estrus, which was immediately before embryo collection in pregnant animals. Each serum sample was evaluated using two ECF test cassettes (tests 1 and 2), and the result of each test cassette was interpreted by two independent readers (readers 1 and 2). Test sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 86, 4, 49, 23, and 46%, respectively. Although the observed agreement between readers (91% for test 1; 89% for test 2) and between tests for the same serum sample (94% for reader 1; 91% for reader 2) was high, the overall rates of false-positive and false-negative ECF test results were 96 and 14%, respectively. We conclude that the ECF test is an unreliable method for determining pregnancy status of dairy cattle on day 6 after estrus.
(**Key words:** early conception factor, pregnancy diagnosis, dairy cattle)

Abbreviation key: ECF = Early Conception Factor, **EPF** = early pregnancy factor, **PPV** = positive predictive value, **NPV** = negative predictive value.

INTRODUCTION

New technologies to identify nonpregnant dairy cows and heifers early post AI may play a key role in a reproductive management strategy for commercial dairy operations. Coupling a nonpregnancy diagnosis with a management decision to quickly reinitiate AI service improves reproductive efficiency and pregnancy rate by decreasing the interval between AI services, thereby increasing AI service rate. Inaccurate diagnosis of nonpregnancy (i.e., false negatives) consequently increases the rate of iatrogenic early embryonic loss when $PGF_{2\alpha}$ or one of its analogues is administered to synchronize estrus behavior or ovulation to hasten the subsequent AI service. Although inaccurate diagnosis of pregnancy (i.e., false positives) is undesirable, its consequences are less severe compared with inaccurate diagnosis of nonpregnancy. Nonetheless, a high rate of false-positive results diminishes the usefulness and cost effectiveness of an early pregnancy test by incorrectly diagnosing animals as pregnant, thereby failing to present a management opportunity to return nonpregnant animals to AI service early post AI and potentially increasing the interval to the subsequent AI service.

Methods developed for early pregnancy diagnosis in dairy cattle must accurately differentiate between pregnant and nonpregnant cows. Cowside milk progesterone assays conducted between 18 and 24 d post AI had a reported accuracy of 97.2% for cows identified as nonpregnant (Pennington et al., 1985), representing the earliest proven method for identifying nonpregnant animals. Recently, a new early pregnancy test has become commercially available for use in cattle. The Early Conception Factor (**ECF**) test (Concepto Diagnostics, Knoxville, TN) reportedly detects a pregnancy-associated glycoprotein within 48 h of conception. Early pregnancy factor (**EPF**) was first identified in pregnant mice (Morton et al., 1987) and later in sheep and cattle (Nancarrow et al., 1981) by using the rosette inhibition test.

Received January 12, 2001.
Accepted April 27, 2001.
Corresponding author: P. M. Fricke, e-mail: fricke@calshp.cals.wisc.edu.

Early pregnancy factor is a secreted substance with growth regulatory and immunomodulatory properties that is required for successful establishment of pregnancy and for proliferation of both normal and neoplastic cells in vivo and in vitro (Cavanagh, 1996). Significant differences in rosette inhibition titer were observed between pregnant and nonpregnant cows on d 13 to 16 and 25 post AI (Sakonju et al., 1993) suggesting that measurement of EPF activity is useful as a method for early pregnancy diagnosis in cattle. Antibodies generated against a glycoprotein immunosuppressive early pregnancy factor isolated from sera of pregnant cows are used to detect EPF in the ECF assay (Threlfall, 1994).

Two studies have compared results from the ECF test conducted between d 3 to 7 and d 11 to 15 post-AI to pregnancy diagnosis using palpation per rectum and ultrasound ranging from 25 to 60 post-AI (Adams and Jardon, 1999; Des Côteaux et al., 2000). One concern with these assessments is that animals with viable embryos during early pregnancy that subsequently undergo embryonic loss before pregnancy diagnosis using palpation per rectum or transrectal ultrasonography increase the rate of false-positive results and bias the assessment. The fertilization rate after AI in beef cows is 90%, whereas embryonic survival rate is 93% by d 8 and only 56% by d 12 post-AI (Diskin and Sreenan, 1980). Similarly, only 48% of embryos recovered from dairy cows on d 7 after AI were classified as normal (Weibold, 1988). Thus, substantial pregnancy loss likely occurred before the establishment of pregnancy status using rectal palpation or transrectal ultrasonography in these studies.

The objective of this study was to evaluate the accuracy of the ECF test and eliminate the likelihood of incorrect false-positive and false-negative results by using serum samples collected from dairy cattle with a known pregnancy status on d 6 after estrus.

MATERIALS AND METHODS

Animals and Estrus Detection

Primiparous and multiparous Holstein cows and nulliparous Holstein heifers were housed at the Dairy Cattle Research Center and the Arlington Dairy Cattle Research Center at the University of Wisconsin-Madison. Animals were managed using standard dairy husbandry practices, and the University of Wisconsin Animal Care and Use Committee approved all animal handling and sample collection procedures.

Cows were observed visually for standing estrus twice daily for 20 min with the aid of a cow androgenized with hormonal ear implants (Synovex H; Fort Dodge Laboratories Inc., Fort Dodge, IA). To identify animals in estrus that failed to stand during the visual observation periods, each cow was fitted with a pressure-activated heat mount detector (Kamar, Kamar Inc., Steamboat Springs, CO) that was observed twice daily. For nulliparous heifers, the only criterion used for determination of estrous behavior was the presence of an activated heat mount detector (Kamar), which was observed once daily. To ensure that an ovulation had occurred, the presence of a corpus luteum was verified for each animal on d 6 after estrus by using an ultrasound machine equipped with a transrectal 7.5 MHz linear-array transducer (Aloka 500V; Corometrics Medical Systems, Inc., Wallingford, CT). Day 0 of the estrous cycle was defined as the day that mounting behavior or an activated heat mount detector was identified.

Pregnancy Status of Animals

Nonpregnant animals included cows (n = 9) and heifers (n = 8) that were not inseminated after estrus. By contrast, pregnant animals were artificially inseminated on the day of estrus (i.e., d 0) with a single straw of frozen-thawed semen. Each straw contained semen from four high fertility bulls that was mixed at the time of collection and packaged and frozen into single 0.5-ml French straws (ABS Global, De Forest, WI). On d 6 after estrus (5 d after ovulation), a blood sample was collected, and embryos then were recovered by using a nonsurgical flushing technique (Elsden et al., 1976). Recovered embryos were evaluated for quality and were graded on a scale of 1 to 5 (1 = excellent to 5 = degenerated) as described previously (Ahmad et al., 1995). Only serum samples from cows (n = 17) and heifers (n = 1) from which at least one embryo of excellent, good, or fair quality was recovered were included in the present study. Cows from which embryos were recovered included dry (n = 8) as well as lactating (n = 9) animals. Of the 18 embryos recovered, 10 graded excellent, 5 graded good, and 3 graded fair.

Collection and Processing of Serum Samples

Serum samples collected from dairy cattle during the summer and winter of 1999 were used for this experiment. Blood samples were collected by coccygeal venipuncture, allowed to clot for 24 h at 4°C and centrifuged, and serum was harvested and stored at −20°C for 6 to 12 mo. Assessment of frozen-thawed serum in the ECF test is acceptable if the frozen serum sample is thawed completely and vortexed so that any sediment at the bottom of the sample tube is completely resuspended (personal communication with Concepto-Diagnostics). Serum samples were subjected to no more than one freeze-thaw cycle during the storage period before thawing for analysis in the present experiment.

Thus, a total of 35 serum samples collected on d 6 after AI and(or) estrus were evaluated in this study. Serum samples collected from noninseminated animals were evaluated as an unequivocal source of sera from nonpregnant animals (n = 17), whereas serum samples collected from animals inseminated after a detected estrus and from which at least one embryo of excellent, good, or fair quality was recovered at a nonsurgical flush 6 d after AI were evaluated as an unequivocal source of sera from pregnant animals (n = 18).

ECF Test Procedure

The ECF test is a colorimetric, qualitative, lateral-flow assay that uses monoclonal and polyclonal antibodies incorporated onto a nitrocellulose membrane. An antibody-gold conjugate is used to detect the presence of the ECF glycoprotein. Each ECF kit contains 25 individually sealed test cassettes with humidity indicators, one pipette, and one bottle of buffer solution. All ECF test kits used for the study were from the same manufacturing lot (# C99029) purchased from a local distributor and were used for this experiment before the expiration date printed on the product label. All ECF kit reagents were stored at 4°C until used to evaluate the serum samples, and all serum samples were analyzed on the same day.

On the day of the assay, serum samples were allowed to thaw completely and equilibrate to room temperature. Each sample was then vortexed until any sediment at the bottom of the sample storage tube was suspended completely. The humidity indicator packaged with each cassette was verified before proceeding with the test, which was conducted explicitly following the manufacturer's instructions outlined in the product insert (Concepto Diagnostics, 1999). Briefly, the dropper pipette provided with each ECF cassette was used to place one drop of serum onto the nitrocellulose membrane through the sample window of the test cassette followed immediately by the addition of 4 drops of the wash buffer provided with the kit. The cassettes then were allowed to incubate at room temperature for 2 h.

ECF Test Evaluation

To evaluate the repeatability of the ECF test, each serum sample (n = 35) was evaluated by using two separate test cassettes (tests 1 and 2). To evaluate repeatability between readers, two independent readers (readers 1 and 2), who were blind to the experimental design, evaluated the result of each cassette at the end of the 2-h incubation period. Instructions for interpreting the ECF test results included in the package insert were read aloud to each reader immediately before they assessed the cassettes. Briefly, each reader was instructed to categorize the result of each cassette into one of three outcomes (pregnant, nonpregnant, or invalid) as follows. The presence of two reddish colored lines, one each in the "C" and "T" window of the cassette, indicates that the cow conceived and is pregnant, whereas the presence of only one reddish colored line in the "C" (control) region of the cassette indicates that the cow did not conceive and is nonpregnant. Discernment of any line present in the "T" (test) region is to be considered a positive outcome despite the faintness of the line. The absence of a discernable line in the control region of the cassette indicates an invalid test.

Data Analyses

Contingency 2 × 2 tables were constructed for each test within each reader to calculate the ECF test sensitivity, specificity, positive predictive value (**PPV**), negative predictive value (**NPV**), and accuracy based on the unequivocal pregnancy status of each animal at the time of blood sample collection (Martin et al., 1987). Test sensitivity was calculated as the proportion of serum samples from pregnant cattle with a positive ECF test result (number of true-positive results/[number of true-positive results + number of false-negative results]), and test specificity was calculated as the proportion of serum samples from nonpregnant cattle with a negative ECF test result (number of true negative results/[number of true-negative results + number of false-positive results]). The PPV of the test was calculated as the probability that a positive ECF test result is from a pregnant animal (number of true-positive results/[number of true-positive results + number of false-positive results]), and the NPV was calculated as the probability that a negative ECF test result is from a nonpregnant animal (number of true-negative results/[number of true-negative results + number of false-negative results]). Test accuracy was calculated as the probability of correctly identifying the pregnancy status of an animal using the ECF test ([number of true-positive results + number of true-negative results]/[number of true-positive results + number of true-negative results + number of false-positive results + number of false-negative results]). Data from all readers and tests were pooled to calculate the overall ECF test sensitivity and specificity. Agreement between readers and between test repeats was calculated as the number of observations in which the readers or tests agreed expressed as a percentage of the total number of observations. Positive and negative predictive values were calculated for various preselected conception rates based

Table 1. Early Conception Factor (ECF) test results for blood sera collected from pregnant and nonpregnant dairy cattle on d 6 after estrus.[1]

			Pregnancy status[2]	
Test	Reader	ECF test result	Pregnant	Nonpregnant
1	1	Pregnant (+)	16	16
		Nonpregnant (–)	2	1
	2	Pregnant (+)	14	17
		Nonpregnant (–)	4	0
2	1	Pregnant (+)	17	17
		Nonpregnant (–)	1	0
	2	Pregnant (+)	15	15
		Nonpregnant (–)	3	2

[1]Serum samples from pregnant (n = 18) and nonpregnant (n = 17) dairy cattle were evaluated using two ECF test cassettes (tests 1 and 2), and the result of each test was interpreted by two independent readers (readers 1 and 2).

[2]For pregnant dairy cattle, sera were collected on d 6 after estrus and immediately before collecting embryos from Holstein cows (n = 17) and heifers (n = 1) inseminated after a detected estrus and in which at least one embryo of excellent, good, or fair quality was recovered at a nonsurgical flush 6 d after AI; for nonpregnant dairy cattle, sera were collected on d 6 after estrus from noninseminated Holstein cows (n = 9) and heifers (n = 8).

on the observed ECF test sensitivity and specificity by using the Epi Info (1997) computer software program.

RESULTS AND DISCUSSION

A novel aspect of the present assessment of the ECF test kit is that serum samples used for this evaluation were collected from dairy cattle with a known pregnancy status on d 6 after estrus. Results from this study, therefore, represent an accurate and unbiased assessment of the accuracy of the ECF test to determine pregnancy status in dairy cattle on d 6 after estrus. Although the ECF test reportedly detects a pregnancy-associated glycoprotein present in bovine serum within 48 h of conception, we chose to assess the ECF test on d 6 after estrus because the corpus luteum is not responsive to $PGF_{2\alpha}$ before d 3 in dairy heifers and d 5 in lactating and nonlactating dairy cows (Momont and Seguin, 1984).

In the present study, results from each of the 70 ECF test cassettes were valid based on a nonactivated humidity indicator packaged with each test cassette and the presence by the end of the 2-h incubation period of a reddish colored line in the control region of each test cassette as assessed by each reader. Results for each ECF test within each reader were compared to the unequivocal pregnancy status of each animal at the time of blood sample collection (Table 1). Pooled results of evaluations for the two tests conducted for each serum sample by each reader resulted in an overall test sensitivity, specificity, PPV, NPV, and accuracy of 86, 4, 49, 23, and 46%, respectively (Table 2).

The test sensitivity, which measures the ability of the test to correctly identify pregnant animals, was 86%, which translates to a 14% incidence of false-negative results. By contrast, the specificity of the test, which measures the ability to correctly identify nonpregnant animals, was 4%, which translates to a 96% incidence of false-positive results. A test that frequently generates positive results regardless of pregnancy status would similarly result in a high sensitivity and a low specificity. Similar but slightly greater sensitivity and specificity results were reported previously (Des Côteaux et al., 2000), and the high rate of false-positive results reported in that study was partially attributed to the likely occurrence of embryonic loss between the time that blood samples were collected on d 11 to 15 post-AI and the final confirmation of pregnancy status which occurred between d 25 and 60 post-AI. This rationale, however, would not explain the rate of false-positive results observed in the present study because pregnancy status at the time of blood collection on d 6 was known for each animal. In addition, the frequency of positive ECF test results increased with increasing incubation times of 30, 60, and 90 min (Des Côteaux et al., 2000), which could partially explain the discrepancy with the present study in which all test results were read after a 120-min incubation period.

In the present study, the observed agreement between readers was 91% (32/35) for test 1 and 89% (31/35) for test 2, whereas observed agreement between tests for the same serum sample was 94% (33/35) for reader 1 and 91% (32/35) for reader 2 (Table 1). Both readers easily distinguished the intensity of the line in the control region of each cassette, whereas the intensity of the line in the test region varied among cassettes from nondiscernible (i.e., no visible line) to a similar intensity to the line in the control region of the cassette. In general, the faintness of the lines in the test region of some cassettes accounted for the discrepancies between readers and tests.

Agreement between readers and tests in the present study could indicate that the test does detect a compound present in bovine serum on d 6 after estrus, but that this compound is not closely associated with pregnancy status. The EPF molecule is secreted during periods of cellular proliferation, peaking during the logarithmic phase of proliferation and decreasing after cellular growth arrest or cellular differentiation (Morton et al., 1987). Furthermore, the amino acid sequence of EPF derived from human platelets is highly homologous to the sequence of rat mitochondrial chaperonin 10 (Cavanagh, 1996), a heat shock protein that functions as a molecular chaperone (Ellis and van der Vies, 1991; Lindquist and Craig, 1988). The principal role of the embryo during maternal recognition of pregnancy

Table 2. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy for Early Conception Factor (ECF) test results.[1]

Test	Reader	Sensitivity[2]	Specificity[3]	PPV[4]	NPV[5]	Accuracy[6]
1	1	89% (16/18)	6% (1/17)	50% (16/32)	33% (1/3)	49% (17/35)
	2	78% (14/18)	0% (0/17)	45% (14/31)	0% (0/4)	40% (14/35)
2	1	94% (17/18)	0% (0/17)	50% (17/34)	0% (0/1)	49% (17/35)
	2	83% (15/18)	12% (2/17)	50% (15/30)	40% (2/5)	49% (17/35)
Pooled results		86% (62/72)	4% (3/68)	49% (62/127)	23% (3/13)	46% (65/140)

[1]Serum samples from pregnant (n = 18) and nonpregnant (n = 17) dairy cattle were evaluated using two ECF test cassettes (tests 1 and 2) and the result of each test was interpreted by two independent readers (readers 1 and 2).

[2]Proportion of serum samples from pregnant cattle with a positive ECF test result.

[3]Proportion of serum samples from nonpregnant cattle with a negative ECF test result.

[4]Probability that a positive ECF test result is from a pregnant animal.

[5]Probability that a negative ECF test result is from a nonpregnant animal.

[6]Probability of correctly identifying pregnancy status.

in cattle is to inhibit the development of endometrial oxytocin receptors, which in turn inhibits endometrial release of $PGF_{2\alpha}$ and luteal regression (Mann et al., 1999). It is well established that bovine embryos must produce a protein called interferon-τ to inhibit luteolysis, thereby establishing maternal recognition of pregnancy (for a review see Mann et al., 1999). Administration of exogenous recombinant bovine interferon-τ to Holstein cows extended luteal lifespan and eliminated oxytocin-induced secretion of $PGF_{2\alpha}$ (Myer et al., 1995). Expression of mRNA for interferon-τ is not detectable until d 12 of gestation in cows, is maximal on d 15 to 16, and continues to be expressed at least until d 25 of gestation (Farin et al., 1990). Thus, maximal expression of interferon-τ mRNA temporally coincides with normal timing of luteal regression in cattle. Based on those reports, further evaluation of EPF, perhaps specifically interferon-τ, as an unequivocal marker for pregnancy in cattle is warranted with more emphasis on detection during d 13 to 17 after insemination.

To simulate the expected performance of the ECF test based on present results, PPV and NPV were calculated for various conception rates observed for herds of lactating dairy cows and dairy heifers based on the ECF test sensitivity and specificity observed in the present study. The PPV increased and NPV decreased as conception rate increased (Table 3). Although the PPV increases as the conception rate in a herd increases, the NPV would be less than 50% (i.e., no better than a guess) in dairy herds exhibiting a conception rate greater than 25%. This simulation illustrates the unreliability of the ECF test as a tool for detection of nonpregnant animals and underscores the potential for iatrogenic embryonic loss if $PGF_{2\alpha}$ or one of its analogues is administered to animals based on a negative ECF test result.

CONCLUSION

The ECF test, in its present form, is an unreliable method for determining pregnancy status on d 6 after estrus in dairy cattle. Although the predictive value of a positive ECF test result increases as the conception rate in a herd increases, the predictive value of a nega-

Table 3. Positive predictive value (PPV) and negative predictive value (NPV) of the Early Conception Factor (ECF) test for selected conception rates[1].

	Conception rate (%)										
Virgin heifers					40	45	50	55	60	65	70
Lactating Cows	20	25	30	35	40	45	50				
PPV (%)	19	24	29	34	39	44	49	54	59	64	69
NPV (%)	69	46	40	35	30	40	35	31	16	13	11

[1]For each conception rate, positive and negative predictive values were calculated based on the observed test sensitivity and specificity of 86% and 4%, respectively.

tive ECF test result would be less than 50% (i.e., no better than a guess) in dairy herds exhibiting a conception rate greater than 25%. Dairy producers who choose to use this commercially available ECF test as a tool for early detection of nonpregnant dairy cattle can expect a high rate of iatrogenic embryonic loss when administering $PGF_{2\alpha}$ or one of its analogues to animals based on a negative ECF test result.

ACKNOWLEDGMENTS

The authors thank Pamela Ruegg and Anne Freeman for their assistance with data analysis for this study. Supported by Hatch project WIS04222 to P.M.F. and a grant from CAPES (Brazil) to R.S.

REFERENCES

Adams, C. S., and P. W. Jardon. 1999. Evaluation of the early conception factor tests in cows 3–7 days post-breeding. Proc. Am. Assoc. Bov. Pract. 32:240–241.

Ahmad, N., F. N. Schrick, R. L. Butcher, and E. K. Inskeep. 1995. Effect of persistent follicles on early embryonic losses in beef cows. Biol. Reprod. 52:1129–1135.

Cavanagh, A. 1996. Identification of early pregnancy factor as chaperonin 10: implications for understanding its role. Rev. Reprod. 1:28–32.

Concepto Diagnostics. 1999. Early conception factor (ECF™) assay kit for cattle, product insert. Concepto Diagnostics, Knoxville, TN.

Des Côteaux, L., P. D. Carrière, and M. Bigras-Poulin. 2000. Evaluation of the Early Conception Factor (ECF) dipstick test in dairy cows between days 11 and 15 post-breeding. Bov. Pract. 34:87–91.

Diskin, M. G., and J. M. Sreenan. 1980. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J. Reprod. Fert. 59:463–468.

Ellis, R. J., and S. M. van der Vies. 1991. Molecular chaperones. Ann. Rev. Biochem. 60:321–347.

Elsden, R. P., J. F. Hasler, and G. E. Seidel, Jr. 1976. Non-surgical recovery of bovine eggs. Theriogenology 6:523–532.

Epi Info Version 6.0. 1997. Centers for Disease Control and Prevention, Atlanta, GA.

Farin, C. E., K. Imakawa, T. R. Hansen, J. J. McDonnell, C. N. Murphy, P. W. Farin, and R. M. Roberts. 1990. Expression of trophoblastic interferon genes in sheep and cattle. Biol. Reprod. 43:210–218.

Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. Ann. Rev. Genetics 22:631–677.

Mann, G. E., G. E. Lamming, R. S. Robinson, and D. C. Wathes. 1999. The regulation of interferon-τ production and uterine hormone receptors during early pregnancy. J. Reprod. Fertil. (Suppl.) 54:317–328.

Martin, S. W., A. H. Meek, and P. Willeberg. 1987. Veterinary Epidemiology, Principles and Methods. Iowa State University Press, Ames, IA.

Momont, H. W., and B. E. Seguin. 1984. Influence of day of estrous cycle on response to $PGF_{2\alpha}$ products: implications for AI programs for dairy cattle. Pages 336.1–336.3 *in* Proc. 10th Int. Congr. on Anim. Reprod. and AI, Univ. of Illinois at Urbana-Champaign, IL.

Morton, H., B. E. Rolfe, and A. C. Cavanagh. 1987. Ovum factor and early pregnancy factor. Curr. Topics Dev. Biol. 23:73–92.

Myer, M. D., P. J. Hansen, W. W. Thatcher, M. Drost, L. Badinga, R. M. Roberts, J. Li, T. L. Ott, and F. W. Bazer. 1995. Extension of corpus luteum lifespan and reduction of uterine secretion of prostaglandin $F_{2\alpha}$ of cows in response to recombinant interferon-τ. J. Dairy Sci. 78:1921–1931.

Nancarrow, C. D., A. L. C. Wallace, and A. S. Grewal. 1981. The early pregnancy factor of sheep and cattle. J. Reprod. Fert. (Suppl.) 30:191–199.

Pennington, J. A., L. H. Schultz, and W. F. Hoffman. 1985. Comparison of pregnancy diagnosis by milk progesterone on day 21 and day 24 postbreeding: field study in dairy cattle. J. Dairy Sci. 68:2740–2745.

Sakonju, S., S. Enomoto, S. Kaminura and K. Amana. 1993. Monitoring bovine embryo viability with early pregnancy factor. J. Vet. Med. Sci. 55:271–274.

Threlfall, W. R. 1994. Immunosuppressive early pregnancy factor (ISEPF) determination for pregnancy diagnosis in dairy cows. Theriogenology 41:317 (Abstr.).

Weibold, J. L. 1988. Embryonic mortality and the uterine environment in first-service lactating dairy cows. J. Reprod. Fert. 84:393–395.

Wiltbank, M. C., P. M. Fricke, S. Sangritasvong, R. Sartori, and O. J. Ginther. 2000. Mechanisms that prevent and produce double ovulations in dairy cattle. J. Dairy Sci. 83:2998–3007.