Article

Evaluation of early conception factor lateral flow test to determine nonpregnancy in dairy cattle

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Abstract — The early conception factor (ECF) lateral flow test was evaluated for its ability to accurately determine nonpregnant status in dairy cattle. Results of 2 field trials involving 191 cows and 832 tests indicated the probability that a cow can be correctly diagnosed as nonpregnant by using the ECF test is only about 50%. Agreement of test results between milk and serum obtained from the same cow was 57.5%. The ECF test was not consistent in identifying nonpregnancy when the same cows were tested repeatedly over a period of 4 weeks. We conclude that the ECF lateral flow test does not accurately identify nonpregnancy in dairy cattle.

Résumé — Évaluation du test du facteur précoce de conception par flux latéral dans la détermination de la non-gestation chez les vaches laitières. Le test du facteur précoce de conception par flux latéral (FPC) a été évalué pour sa capacité à déterminer de façon précise l'état de non-gestation des vaches laitières. Les résultats de 2 essais sur le terrain comprenant 191 vaches et 832 tests ont indiqué que la probabilité qu'un vache soit correctement diagnostiquée non gestante par le test FPC n'est que d'environ 50 %. La concordance des résultats des tests effectués sur le lait et le sérum de la même vache était de 57,5 %. Le test du FPC n'était pas constant dans la reconnaissance de la non-gestation lorsque les mêmes vaches étaient testées à répétition sur une période de 4 semaines. Nous concluons que le test du FPC par flux latéral ne réussit pas à identifier précisément la non-gestation chez les vaches à lait.

(Traduit par Docteur André Blouin)

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Introduction

Reproductive inefficiency in dairy cattle is a major economic problem for dairy producers. More than 30% of the cows removed from dairy herds each year in Western Canada are culled due to reproductive problems (1). Further, the average 1st service conception rate to artificial insemination is only about 40%. If a cow is bred but does not conceive, she should return to estrus in approximately 21 d. However, since estrus detection is less than optimal in modern dairy herds, many cows that fail to conceive are not identified until a veterinarian performs pregnancy diagnosis at about 40 d after breeding. Based on 1 estimate (2), up to $230 million in potential income is lost annually by Canadian dairy farmers, because the current national average calving interval is 14 mo (1). Therefore, any technology that allows an accurate determination of nonpregnancy in less than 21 d after insemination should help to improve reproductive efficiency in dairy cattle.

The early conception factor (ECF) lateral flow test (Concepto Diagnostics, Knoxville, Tennessee, USA) has been marketed as a tool that allows the identification of nonpregnant cows from 6 d after breeding (3). The ECF test for cattle is reportedly designed to detect the presence of a protein, designated early conception factor, that becomes detectable in blood of pregnant cows as early as 48 h after mating (4), remains detectable throughout gestation (4,5) and declines quickly following embryonic death. According to some reports, the test is over 94% accurate in identifying nonpregnant cows (3–5). However, other reports have indicated that the ECF test is not a good diagnostic test for determining nonpregnancy (6,7). There is a growing interest among dairy producers in using the ECF test as a reproductive management tool. Many dairy practitioners are also curious about the efficacy of this test.

The overall objective of this study was to evaluate the efficiency of the ECF test in accurately determining nonpregnancy

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This study was jointly funded by the Alberta Agricultural Research Institute, Alberta Milk, and Alberta Agriculture Food, and conducted between 1999 and 2001. The ECF test kits were either donated (Trial A) or made available at a discounted bulk price (Trial B) by Concepto Diagnostics, Knoxville, Tennessee, USA. According to the information available at the time of writing this report, Concepto Diagnostics was acquired by EDP Biotech Corporation (Knoxville, Tennessee) in August 2005, and the ECF test is currently being marketed as the EDP/ECF test.

in dairy cattle, and to develop recommendations, based on the findings, on the suitability of the ECF test as a tool for reproductive management of dairy cattle. Specific objectives were as follows: 1) to determine the negative predictive value of the ECF test (the probability that a negative ECF test was from a nonpregnant cow) for early determination of nonpregnancy in dairy cattle, using a sample of milk or serum; 2) to determine the specificity (ability to correctly identify nonpregnant cows) of the ECF test; 3) to evaluate the ECF test for consistency (repeatability) in identifying a nonpregnant cow when used at various times over a period of 4 wk; 4) to evaluate between-assessor agreement when 2 individuals evaluated results of the test; and 5) to evaluate between-sample agreement when the test was applied to milk and a blood sample from the same animal.

Materials and methods

Two separate trials involving 191 cows were conducted. Trial A involved 28 cows; trial B involved 163 cows. Five commercial dairy herds and the research herd at the University of Alberta were involved in this project. Animals were housed and cared for according to the guidelines of the Canadian Council on Animal Care (8). Experimental procedures were preapproved by the Animal Welfare and Policy Committee, University of Alberta. For the practicality of having access to a sufficient number of animals during each test day, the ovarian status of cows was synchronized by using an established protocol (9) that constituted 2, 100 μg injections of gonadotropin releasing hormone (gonadorelin) (Fertiline; Vetoquinol NA, Lavaltrie, Quebec) given, IM, 9 d apart, and a single 25 mg injection of prostaglandin $F_{2\alpha}$ (dinoprost) (Lutalyse; Pfizer Animal Health, Orangeville, Ontario) given, IM, 7 d after the 1st gonadorelin treatment. All cows were inseminated 16 to 20 h after the 2nd gonadorelin treatment.

Trial A

A sample of milk and 1 of blood were obtained from 28 cows in the research herd on the day of anticipated estrus (Day 0), immediately prior to insemination, and then at 7, 14, 21, and 28 d after insemination. Samples were handled and tested as per ECF test directions (3).

Blood samples were allowed to clot at room temperature for 2 to 3 h for serum separation. Milk samples were either refrigerated or stored on ice and tested within 3 h of collection. The ECF test kits, consisting of test cassettes, droppers, and buffer solution, were stored at 4°C and allowed to reach room temperature before use. Cassettes were placed on a flat surface and labeled with the cow number, experimental day, and sample type. Specified quantities of the test sample (serum or milk) and buffer solution were placed on the ECF cassette as directed (3). Cassettes remained undisturbed for a minimum of 2 h before results were evaluated. The appearance of a single red line against the letter "C" (control) was indicative of a valid negative test. The appearance of a red line against "C" and a 2nd red line against the letter "T" was indicative of a valid positive test. The nonappearance of a red line against "C" or the appearance of only a single red line against "T" was considered an invalid test. To determine between-assessor agreement of test results, results of the ECF test from a subset of 10 cows were evaluated by 2 individuals and recorded independently. All other evaluations were done by 1 person only.

Table 1. False negative results (%) from the early conception factor test when milk or serum samples were tested in Trial A

		False negative (%)	
Day	*n*	Milk	Serum
7	27	55.0	42.9
14	26	50.0	40.0
21	27	58.8	50.0
28	26	43.8	25.0

Pregnancy diagnosis was performed by transrectal ultrasonography 28 d after insemination: visualization of a fluid-filled amniotic vesicle and the fetus was considered to be positive for pregnancy. The outcome of the ultrasonography-based pregnancy diagnosis was used as the "standard of validity" to declare a cow as being either "truly pregnant" or "truly nonpregnant" on the day of pregnancy diagnosis.

Test results from milk and serum samples from all 28 cows were used to calculate the repeatability of negative results (ability to consistently identify a nonpregnant cow as nonpregnant every time) when tested over a period of 4 wk. Negative predictive value, positive predictive value, test specificity, test sensitivity, and accuracy were determined, as described by Smith (10). Truly pregnant cows are those that are confirmed pregnant by ultrasonography or transrectal palpation. Truly nonpregnant cows are those that are confirmed nonpregnant by ultrasonography or transrectal palpation. Test positive means that the ECF test result is indicative of pregnancy. Test negative means that the ECF test result is indicative of nonpregnancy. When test results are positive but the cows are diagnosed as nonpregnant, such cases are regarded as false positives. False negatives refer to cases in which test results are negative, but the cows are diagnosed as pregnant. When both test results and pregnancy diagnosis are in agreement that the cows are nonpregnant, such cases are referred to as true negatives. Test sensitivity is defined as the likelihood of obtaining a positive ECF test result in cows that are truly pregnant. Test sensitivity is calculated as the percentage of serum or milk samples from pregnant cattle with a positive ECF result [true positives/(true positives + false negatives)]. Sensitivity of the test does not apply to samples obtained on Day 0 (preinsemination; no true positives) and, therefore, is not calculated. Test specificity is defined as the likelihood of obtaining a negative ECF test result in cows that are truly nonpregnant. In other words, test specificity is the percentage of serum or milk samples from nonpregnant cattle with a negative ECF result [true negatives/(true negatives + false positives)]. Positive predictive value is the probability that a positive ECF result is from a pregnant cow [true positive/(true positive + false positive)]. The positive predictive value of the test does not apply to samples obtained on Day 0 (preinsemination; no true positives) and, therefore, is not calculated. Negative predictive value of the test is the probability that a negative ECF test is from a nonpregnant cow [true negatives/(true negatives + false negatives)]. Test accuracy is defined as the proportion of all tests, both positive and negative, that are correct. Test accuracy

Table 2. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of early conception factor (ECF) test results, using milk samples (Trial A)

	Day of gestation	*n*	Sensitivity[a]	Specificity[b]	PPV[c]	NPV[d]	Accuracy[e]
Preinsemination	0	28	na	65%	na	100%*	65%
Postinsemination	7	27	21%	69%	43%	45%	44%
	14	26	46%	54%	50%	50%	50%
	21	26	23%	54%	33%	41%	38%
	28	26	46%	69%	60%	56%	58%

[a] Proportion of pregnant cows with a positive ECF result
[b] Proportion of nonpregnant cows with a negative ECF result
[c] Probability that a positive ECF result is from pregnant cows
[d] Probability that a negative ECF result is from nonpregnant cows
[e] Probability of identifying pregnancy status correctly using ECF results
na = not applicable
* The NPV of 100% should not be misinterpreted because NPV on Day 0 can only be 100% as there could be no false negatives on Day 0; note that the test accuracy is only 65%

Table 3. Evaluation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of early conception factor (ECF) test results, using serum samples (Trial A)

	Day of gestation	*n*	Sensitivity[a]	Specificity[b]	PPV[c]	NPV[d]	Accuracy[e]
Preinsemination	0	28	na	25%	na	100%*	25%
Postinsemination	7	27	79%	31%	55%	57%	56%
	14	26	85%	23%	52%	60%	54%
	21	26	77%	23%	50%	50%	50%
	28	26	93%	23%	57%	75%	59%

[a] Proportion of pregnant cows with a positive ECF result
[b] Proportion of nonpregnant cows with a negative ECF result
[c] Probability that a positive ECF result is from a pregnant cow
[d] Probability that a negative ECF result is from a nonpregnant cow
[e] Probability of identifying pregnancy status correctly using ECF results
na = not applicable
* The NPV of 100% should not be misinterpreted because NPV on Day 0 can only be 100% as there could be no false negatives on Day 0; note that the test accuracy is only 25%

is the probability of identifying correctly the pregnancy status of an animal when using ECF test results [true positives + true negatives/(true positives + true negatives + false positives + false negatives)].

Contingency 2 × 2 tables were constructed for each test within each assessor and for each test material (milk vs serum) over the sampling time periods. All calculated values were determined over test interval and material used (milk or serum). Between-assessor agreement was determined by using Categorical Response Model (PROC CATMOD) and Frequency Procedures (PROC FREQ) of the SAS software (SAS Institute, Cary, North Carolina, USA). Chi-square tests were applied and Kappa agreement coefficients generated. The contingency tables were used to calculate the ECF test sensitivity, specificity, positive predictive value, negative predictive value, and accuracy.

Trial B

One hundred and sixty-three dairy cows from participating commercial herds in Alberta were used. Milk and blood samples were obtained from each cow; 1st sample (n = 163) at 14 d after insemination, and a 2nd sample (n = 131) at the time of pregnancy diagnosis (palpation per rectum) by the herd veterinarian, 40 ± 6 d after insemination. Based on the veterinarian's diagnosis, cows were declared "truly pregnant" or "truly nonpregnant," and these results were used as the "standard" for comparison with the ECF test results. Samples were handled and tests conducted as per instructions (3). Test results from whole milk and serum samples were used to calculate negative predictive value, positive predictive value, test specificity, test sensitivity, and accuracy at Days 14 and 40 ± 6 d after insemination.

Test results from Trials A and B obtained on Day 14 after insemination (188 milk and blood samples, after excluding 3 invalid results) were used to determine between-sample agreement.

Results

Trial A

False negative results were high on Days 7, 14, 21, and 28 after insemination (Table 1), regardless of the type of sample tested. False negative results pertaining to Day 0 are not reported because all cows were truly nonpregnant at that time (samples obtained prior to insemination) and there could be no false negatives on Day 0.

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the ECF test at the various test days pertaining to milk and serum samples are presented in Tables 2 and 3, respectively. Results of samples obtained on Day 0 (prior to insemination) indicate clearly that the test is

Table 4. Between-assessor agreement of early conception factor (ECF) test results for serum and milk collected from Holstein dairy cattle ($n = 10$) in Trial A. The Kappa agreement coefficient (κ) for each sample type (milk or serum) and its probability of significance appear as footnotes

Sample	*n*	Assessor 1 (+)	Assessor 1 (−)	Assessor 2 (+)	Assessor 2 (−)	Disagree *n* (%)	Agree *n* (%)
Milk	43	32	11	38	5	6 (14)	37 (86)[a]
Serum	44	11	33	9	35	2 (4)	42 (96)[b]

[a] $k = 0.13$, $P = 0.10$
[b] $k = -0.05$, $P = 0.61$

unreliable, as it was not able to correctly identify truly nonpregnant cows. Only 17 of 28 milk samples and 6 of 28 serum samples taken on Day 0 (preinsemination) tested negative to ECF.

While testing for repeatability (the ability to consistently identify a truly nonpregnant cow as nonpregnant at each time tested), only 2 of the 13 nonpregnant cows consistently tested negative to ECF at all 5 times (serum test), and were later confirmed nonpregnant by ultrasonography. When the milk sample was used, none of the 13 nonpregnant cows was consistently identified as nonpregnant by the ECF test. Based on observations of 2 independent assessors on the subset of 10 cows, between-assessor agreement was calculated to be 86% and 96% for milk and serum samples, respectively (Table 4). Results did not differ ($P \geq 0.10$) between assessors for both milk and serum ECF tests. The Kappa agreement coefficients and their probabilities of difference are also reported in Table 4.

Trial B

As observed in Trial A, false negative results were high on both test days (Table 5), regardless of the type of sample tested. Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the ECF test based on data obtained from all cows in Trial B are presented in Table 6.

The percent agreement between results of milk and serum tests performed 14 d after insemination was determined to be 60%.

Overall, only 8 (less than 1%) of the 832 ECF test cassettes yielded an invalid result wherein the control "red line" did not appear at the point marked C. Invalid test results were excluded from all analyses. The overall conception rate (including cows in both trials) to the timed insemination program was 46.6%.

Discussion

The rosette inhibition test has been used to determine the presence of early pregnancy factors in livestock species, including cattle, with an accuracy of over 90%, as described previously (11). However, because the rosette inhibition test is not suitable for cow-side applications and is time-consuming, alternative methods have been explored. Even though the ECF test is advertised as having high precision for determining nonpregnant status in dairy cattle, no scientific reports to confirm this were available at the time of initiation of this study. Considering that the test is being marketed as a product to determine nonpregnancy in cattle, negative predictive value and test specificity values will be the main focus of this discussion.

Table 5. False negative results (%) from early conception factor test when milk or serum samples were tested in Trial B

Day	*n*	False negative (%) Milk	False negative (%) Serum
14	163	42.1	50.0
41	131	41.2	37.5

The between-assessor agreement (Trial A) was greater for serum (96%) than for milk (86%), similar to the findings of Cordoba et al (12), who reported between-assessor agreement in the range of 89% to 91%. Thus, it appears that when 2 individuals evaluate the same test, their results will agree most times, although assessors' results were more alike for serum samples. Repeatability of a negative test (the ability to consistently identify a truly nonpregnant cow as nonpregnant when tested at different times) was very low.

In Trial A, samples were obtained from cows prior to insemination and used as "negative control," because there is no possibility for the presence of the ECF protein in the serum or milk of noninseminated cows. Thus, it was expected that the ECF test would correctly identify these samples with a negative result yielding a very high test-specificity on Day 0. Contrary to our expectation, the specificity of the test for the preinsemination milk and serum samples was unacceptably low at 65% and 25%, respectively. Even though the negative predictive value was 100% (on Day 0 preinsemination samples) it has little value, because 35% and 75% of the cows were not correctly identified as nonpregnant by the ECF test when milk or serum was used.

Similarly, the probability of identifying pregnancy status correctly, using ECF test result on Days 7, 14, 21, or 28 postinsemination, ranged from 38% to 59%, with an overall average of less than 55%, regardless of the test material used. The specificity and the negative predictive value on postinsemination days were also unacceptably low.

The greatest value of any test to the producer comes from its ability to accurately identify nonpregnant cows within 21 d after breeding. It has previously been reported that the ECF test is over 94% accurate in determining nonconception in dairy cattle (4,5). Contrary to these reports, the accuracy of the ECF test in identifying nonpregnant cows at 7, 14, or 21 d after breeding in the present study was poor, ranging from 38% to 51%. It may be argued that a few of these cows could have been pregnant at test times (Day 7, 14, or 21) but eventually lost the pregnancy due to embryonic death. However, this argument cannot be supported, because the ECF test results were equally inaccurate, even on the day of pregnancy confirmation (Day 28 or later) when there was no question about the pregnancy status. Threlfall and Bilderbeck (4) found that the accuracy of the ECF test increased with gestation and became 100% accurate at 8 mo. The present study did not find such evidence, as the test accuracy ranged from 38% to 61% at all test times, regardless of the stage of gestation (range, 7 to 46 d).

The low specificity and negative predictive value of the ECF test results obtained in Trial A were reconfirmed in Trial B,

Table 6. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of early conception factor (ECF) test results from Trial B

Day of gestation	*n*	Material	Sensitivity[a]	Specificity[b]	PPV[c]	NPV[d]	Accuracy[e]
14	163	Milk	67%	37%	47%	58%	51%
40 ± 6	131	Milk	76%	29%	48%	59%	61%
14	163	Serum	92%	7%	45%	50%	45%
40 ± 6	131	Serum	95%	7%	48%	63%	48%

[a] Proportion of pregnant cows with a positive ECF result
[b] Proportion of nonpregnant cows with a negative ECF result
[c] Probability that a positive ECF result is from a pregnant cow
[d] Probability that a negative ECF result is from a nonpregnant cow
[e] Probability of identifying pregnancy status correctly using ECF results

which involved a larger population of cattle. The poor specificity of the ECF test has been reported in 2 other studies (12,13). The poor accuracy and negative predictive value of the tests obtained in the present study are also in agreement with the findings of Cordoba et al (12) and Gandy et al (13).

Results of milk and serum ECF tests agreed only 60% of the time when Day 14 data were considered, which was quite similar to the 63% agreement reported in another study (13).

Findings of the present study indicate that the ECF test result is correct only about 50% of the time, regardless of whether the test result is negative or positive. In other words, the negative predictive value and positive predictive value of the ECF test are approximately equal at about 50%. Therefore, the test offers no information about the true pregnancy status of cows and the use of the test cannot be supported. A recent study evaluated the ECF test on 48 heifers and reported positive and negative predictive values of 60% and 62.5%, respectively (14). The percentage of false-negative results was 66.7%, considerably higher than in the present study, suggesting that even the newer ECF tests are unreliable.

It is concluded that the ECF lateral flow test, as evaluated, has no value as a reproductive management tool, as it does not accurately identify nonpregnancy in dairy cattle.

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