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# Use of early conception factor test for determining pregnancy and embryonic mortality status of dairy cows

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Summary

**The aim of this study was to evaluate the accuracy of the ECF test for detecting the pregnancy status and embryonic mortality and to compare the reliability of ECF test from among ultrasonography and serum progesterone level. In this study, two groups were designed: the study group (n = 15) and control group (n = 9). All cows were observed for estrus activity four times daily. Cows in the study group were inseminated. After insemination, at the 7, 20, 30 and 45$^{th}$ days ECF test and ultrasonographic examination were applied to check the pregnancy status. Cows in the control group were not inseminated and examination procedure was performed like in the study group. Twenty days after insemination, pregnant positive cows that had been determined by ultrasonography were designated the study group. Twenty days after insemination, ECF test were applied and progesterone levels were determined in the serum samples obtained from pregnant positive cows. Fifteen cows in the study group were checked 20 days after insemination and determined pregnant. Their pregnancy status was confirmed 20 days after insemination by using ultrasonography. In the 30$^{th}$ and 45$^{th}$ days ultrasonography was repeated, after which 13 cows were determined pregnant. In the serum of these two cows progesterone levels fell under 2 ng/ml. However, in the 20$^{th}$ day these cows' progesterone levels was higher than 2 ng/ml, in two cows embryonic death occurred. In cows which were determined as pregnant by ultrasonography at the 20$^{th}$ day, the ECF test was applied at the 7$^{th}$ day and 10 cows from this group had a positive reaction (66.7%). Test specificity, PPV and NPV results were 44.4%, 66.7% and 44.4% respectively; at the 20$^{th}$ day the ECF test was positive for 9 cows (60%), specificity, PPV and NPV results were 33.3%, 60.0% and 33.3%; at the 30$^{th}$ day, the ECF test was positive for 12 cows (92.3%), test specificity, PPV and NPV results were 45.5%, 66.7% and 83.3%; at the 45$^{th}$ day, 10 cows (76.9%), test specificity, PPV and NPV results were 54.5%, 66.7% and 66.7% respectively. Between the study groups, the ECF test accuracy at the 7$^{th}$ and 20th days were found lower than at the other days. The test's accuracy was determined the highest at the 30$^{th}$ day (70.8%), and the lowest at the 20$^{th}$ day (50%). The results show that ECF test is an unreliable method for pregnancy diagnosis and for determining embryonic death in dairy cows and these data indicate that the current ECF test cannot accurately identify the nonpregnant cows.**

**Keywords: early conception factor, cow, pregnancy**

New technologies to identify nonpregnant dairy cows and heifers early post artificial insemination (AI) may play a key role in a reproductive management strategy for commercial dairy operations (2). 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 (3).

Methods developed for early pregnancy diagnosis in dairy cattle must accurately differentiate between pregnant or nonpregnant cows (7). Available techniques for the detection of pregnancy in cattle include hormonal assays such as progesterone, pregnancy-specific protein B, early conception factor, estrone sulfate, transrectal palpation and ultrasonography (5).

Palpation of the uterine contest rectally is probably the most commonly used method for pregnancy diagnosis. Pregnancy diagnosis after insemination can be conducted as early as 30 days in heifer and 35 days in cows, although much practice is necessary in order to determine pregnancy at that stage. Rectal palpation has the advantage of beign an accurate, fast, realtively cheap method that is less

labor intensive as compared to the other methods. Nonetheless, training is necessary and exam should be conducted by a veterinarian (2).

Progesterone is the hormone also referred to as the pregnancy hormone. To use the milk progesterone test as a pregnancy indicator the milk and blood samples must be collected between 21 to 24 days after the cow was in estrus and inseminated (2). Progesterone assays conducted between 18 and 24 days post AI had a reported accuracy of 97.2% for cows identified as nonpregnant (8), representing the earliest proven method for identifying nonpregnant animals (2). Low progesterone would indicate that the cow is not pregnant and high progesterone would indicate that the cow has a functional corpus luteum and might be pregnant. Therefore, the test is most accurate in determining that a cow is not pregnant, because if the progesterone levels are low she cannot be pregnant. Reasons progesterone levels might be high between 21 and 24 days after insemination include: the cow is pregnant. The cow is the middle of her estrous cycle but not pregnant, embryonic mortality, abnormalities, such as pyometra. Reasons for this include: variation in estrus cycle length between cows, estrus detection errors, uterine disease (pyometra), ovarian dysfunction and early embryonic mortality. The reliability of progesterone for the diagnosis of pregnancy is not satisfactory in it. Ultrasonography has been reported to detect pregnancy in cattle as early as 9 or 12 days into gestation. But authors recommend that, accuracy of diagnoses improves, however, by Day 25 and 30. The main advantages of the use of ultrasound for pregnancy diagnosis are the high accuracy of the results that are generated and, the fact that pregnancy diagnosis may be conducted relatively early after insemination. The main disadvantages of the use of ultrasonography are related to cost and time involved with the use of this technics. Also the training of the operator needed to interpret the imagines also can serve as a disadvantage (2). While current methods for pregnancy diagnosis are very effective, they are usually performed after implantation has taken place and therefore can rarely be used to discriminate between fertilization failure, which results in a nonpregnant animals, and that which may be due to early embryonic loss post-conception. The ability to detect conception and conception failure post-breeding would be benificial to producers if such a test were specific to early embryonic development and can provide a timely and accurate diagnosis (5).

Recently, a new early pregnancy test has become commercially available for use in cattle. The Early Conception Factor (ECF) test reportedly detects a pregnancy--associated glycoprotein within 48 h of conception (2). The use of EPF as a diagnostic tool requires two components: EPF-A, which is produced by the oviduct during proestrus and estrus also during pregnancy; and EPF-B, which is produced by a local ovary once a local signal from fertilized ovum is present. The apparent role of EPF is as an immunomodulator which may aid in defending the embryo from immunological rejection by the maternal host, and is present as early as 24 h after mating until parturation. When embryo/fetal mortality occurs, or if the fetus removed, EPF decreases within hours thus illustrating the specificity of EPF to conception and pregnancy (5).

Early experiments demonstrated that in cattle fertilization rates were usually high and that embryonic mortality was the main source of reproductive wastage. Progesterone measurements were used in cattle to determine the respective frequently of early and late embryonic mortalities. In the field studies, the use of pregnancy spesific proteins in combination with progesterone measurements is useful to identify the factors (environmental/genetic) influencing specifially the frequencies of early and/late embryonic mortality (6).

The objectives of this study were to evaluate the accuracy of the ECF test for detecting the pregnancy status and embryonic mortality in cows, and to compare the reliability of ECF test among ultrasonography and serum progesterone level.

## Material and methods

The material of the study were involved totally 24 cows of two different breeds (Holstein and Simmental). Age and parity of experimental cows ranged from 1.5 to 9 years and 1 to 7, respectively. The service period of the cows was 92.2 days, on an average. Throughout the experimental period the cows were housed in semi-covered sheds under similar conditions of feeding management. Each cow was fed 25 to 40 kg of green fodder and 3 to 5 kg of a concentrate mixture. Body condition scores (BCS) were evaluated by the same person as described by Edmonson et al. (4). Scores were assigned using a five point scale (0 = very thin to 5 = grossly fat). All the cows in the groups had an optimal BCS (comprised between 2.75 and 3.50). The cows were machine-milked twice daily.

In this trial two groups were designed. First one was a study group (n = 15), second one was a control group (n = 9). All cows were observed for estrus activity four times daily. As a result of vaginal inspection, rectal and ultrasonographic examination, it was confirm that cows were in estrus. Cows in the study group were inseminated. ECF test is used on day 7 or 8 after breeding, as stated by manufacturing company. After insemination in 7, 20, 30 and 45$^{th}$ days ECF test and ultrasonographic examination applied to check of pregnancy status, changes of uterus and structures on ovary. To evaluate changing in uterus and structures on ovary ultrasonographic examination performed in 7 days, although to evaluate embryonic death ultrasonographic examination performed in 30 and 45$^{th}$ days.

Cows in the control group were not inseminated and examination procedure was performed like study group. At the same time in mentioned days to investigate progesterone level in serum, blood samples were collected by juguler venipuncture, allowed to clot and centrifuged and serum was harvested and stored at –20°C.

At 20 days after insemination, pregnant positive cows determined by ultrasonographic examination were designed study group. At 20 days after insemination, ECF test were applied and progesterone level were determined in the serum samples obtained from pregnant positive cows. ECF test and ultrasonographic examination results and progesterone levels were compared between groups.

The ECF test is a colorimetric, qualitative, lateral flow assay that uses monoclonol and polyclonal antibodies incorporated onto a nitrocellulose membrane. Antibody-gold conjugate is used to detect the presence of the ECF glycoprotein. Blood was centrifuged using serum tube. Fresh serum samples were used to determine ECF. Frozen serum was not used in ECF test because inaccurate results might be seen. The humi-

**Tab. 1. The rates of mean $P_4$ (ng/ml), sensitivity (Sen), specificity (Spe), positive predictive value (PPV), negative predictive value (NPV), accuracy, false negative (FN) and false positive (FP) by Early Conception Factor (ECF) test in study group and control group**

| Days | Study Group Mean $P_4$ (ng/ml) | Control Group Mean $P_4$ (ng/ml) | ECF test Sen (%) | ECF test Spe (%) | ECF test PPV (%) | ECF test NPV (%) | ECF test Accuracy (%) | ECF test FN (%) | ECF test FP (%) |
|---|---|---|---|---|---|---|---|---|---|
| 7 | 4.61 ± 3.11 | 3.71 ± 4.43 | 10/15 (66.7) | 4/9 (44.4) | 10/15 (66.7) | 4/9 (44.4) | 14/24 (58.3) | 33.3 | 55.6 |
| 20 | 8.50 ± 5.41 | 2.70 ± 1.49 | 9/15 (60.0) | 3/9 (33.3) | 9/15 (60.0) | 3/9 (33.3) | 12/24 (50.0) | 40.0 | 66.7 |
| 30 | 8.65 ± 5.35 | 6.25 ± 6.52 | 12/13 (92.3) | 5/11 (45.5) | 12/18 (66.7) | 5/6 (83.3) | 17/24 (70.8) | 7.7 | 54.5 |
| 45 | 10.51 ± 6.41 | 6.45 ± 10.75 | 10/13 (76.9) | 6/11 (54.5) | 10/15 (66.7) | 6/9 (66.7) | 16/24 (66.7) | 23.1 | 45.5 |
| Average | 8.29 ± 5.48 | 4.82 ± 6.64 | 41/56 (73.2) | 18/40 (45.0) | 41/63 (65.1) | 18/33 (54.5) | 59/96 (61.5) | 26.8 | 55.0 |

dity 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. The dropper pipette provided with each ECF casette was used to place one drop of serum in the sample window of the test cassette followed by the addition of two drops of the specific serum wash buffer provided in the kit. The cassettes then were allowed to incubate at room temperature. The end of that time; the presence of only red line in the Control (C) area was indicated that the cow did not conceive whereas the presence of two red lines, one in each of the Control (C) and Test (T) area were indicated that the cow conceived.

Progesterone ($P_4$) levels were determined using a commercial RIA kit (Immunotech, Marseille, France). Intra- and interassay coefficients of variation (CV) for the $P_4$ assays were 5.4% and 9.1%, respectively. The thawing serum and standard solution which was in kits were transferred 50 µl into the tubes. And on the mixture, fluid which was contain 500 µl iodine 125 ($I^{125}$) signed antigen was added. Serum samples were vortexed 1 h and incubated in shaker along an hour. End of this time, fluid part was aspirated and the values were read at Gamma Counter. The values were then converted into ng/ml through a computer program (Berthold Immunoprocessing System, version 3.00).

Test sensitivity was calculated as the proportion of serum samples from pregnant cattle with the a positive ECF test results (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 result (number of true negative results/(number of true-negative results + number of false-positive results)), and the positive predictive value (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)), the negative predictive value (NPV) was calculated as the probability that a negative ECF test result is from a non-pregnant animal (number of true-negative results/(number of true-negative results + number of false-negative results)) and 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)) as described by Cordoba et al. (3).

## Results and discussion

Fifteen cows in study group controlled 20 days after insemination and determined pregnant (This group included by pregnancy positive cows). Their pregnancy status accurated 20 days after insemination by using ultrasonographic examination. In the 30th and 45th days ultrasonographic examination repeated then 13 cows determined pregnant. In these two cows serum progesterone levels fell under 2 ng/ml. Although in 20th day this cows progesterone levels higher than 2 ng/ml, but also at ultrasonographic examination, embryos were not determined. The results were shown that, in two cows the embryonic death occurred.

Pregnancy determined by ultrasonographic examination at 20th day (n = 15), ECF test applied at 7th day and ten cows were positive (66.7%). The test specificity, PPV and NPV were 44.4%, 66.7% and 44.4%, 20th day ECF test was positive at 9 cows (60%) and test's specificity, PPV and NPV were 33.3%, 60.0% and 33.3%. At 30th day pregnancy determined by ultrasonografic examination (n = 13) and ECF test was positive at 12 cows (92.3%). The test's specificity, PPV and NPV were 45.5%, 66.7% and 83.3%, at 45th day pregnancy status determined ultrasonografic examination (n = 13) and ECF test was positive at 10 cows (76.9%). The tests specificity, PPV and NPV results were 54.5%, 66.7% and 66.7% respectively (study results was summarised in the table 1).

Between study groups ECF test's accuracy at 7th and 20th days were found lower than the other days. The test's accuracy rate was the highest at 30th day (70.8%), the lowest at 20th day (50%). When ECF test results are critised it is fixed that, in applied days false positive rations higher than false negative rations.

Coupling a nonpregnancy diagnosis with a management decision to quickly reinitiate AI services improves reproductive efficiency and pregnancy rate by decreasing the interval between AI services, thereby increasing AI service rate. This could occur through application of EPF testing programs after breeding in conjuction with PG 7 to 9 day later for accelerating the synchronization of estrus after a conception failure (identified by EPF assays), thus decreasing the number of days a cow remains nonpregnant (5).

A study by Gandy et al. (5) reported that the objectives of their study were evaluate the effectiveness of the ECF test for detecting the nonpregnant cow, and compare the reliability of serum versus milk ECF tests relative to actual pregnancy rates. In this study Holstein Heifers were bred after observed estrus and serum ECF tests conducted between Days 7 and 9 after AI. In this study 55.6% of the confirmed nonpregnant heifers were identified correctly by serum ECF analysis at days 7 to 9 post-AI. Same researchers in this study, 40 lactating cows were synchronized, the animals were bred (AI), and serum and milk ECF tests were performed on Days 3, 9, 15, 21 and 30 after AI. Pregnancy diagnosis (ultrasound on Day 30 and palpation on Day 51) confirmed that 50% of the cows were pregnant to AI, while serum and milk ECF analysis indicated a 100% and 37.5% predicted pregnancy rate, respectively at 30 days post-AI. Moreover, results of the serum and milk ECF disagreed with one another 36.9% of the time overall, while agreement between ECF and actual pregnancy rates were 50.6% and 45.6% for milk and serum respectively. Additionally in this trial, a negative ECF test result only identified 5% and 28.8% of nonpregnant cows overall for serum and milk tests respectively (i.e., true negatives), with a high incidence of false positive ECF results noted (47.5% and 31.3% for serum and milk, respectively).

A study which was performed by Adams and Jardon (1) reported sensitivity and specificity of the ECF test were 51 and 55% respectively. The predictive value of a positive test was 37%, and that for a negative test was 69%. Fourty-nine% of the rectally diagnosed pregnant cows were diagnosed as open by the ECF test. Only 37% of the cows diagnosed pregnant via the ECF test were pregnant on rectal palpation at 41-59 days post-breeding.

A trial performed by Cordoba et al. (3) one concern with previous assesments of ECF 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 asssesment. To preclude the possibility, noninseminated Holstein cows and heifers were evaluated as an unequivocal source of nonpregnant animals, and Holstein cows and heifers inseminated at estrus in which at least one embryo of transferable quality was recovered at a nonsurgical flush 6 day after AI were evaluated as an unequivocal source of pregnant animals. Each serum sample was evaluated using ECF test. Test sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 86, 4, 49, 23, and 46% respectively. Reseachers reported that the ECF test is an unreliable method for determining pregnancy status of dairy cattle on day 6 after estrus.

As our results are shown, ECF test specificity is higher than Cordoba et al. (3), and lower than Adams and Jardon (1). PPV, NPV and accuracy rate are different from Cordoba et al. (3) at 7 days after insemination. Threlfall and Bilderback (10) studies characterizing the ECF test have suggested an accuracy for detecting the nonpregnant cow of 94.5% within 24-48 h after breeding to 100% later in gestation. However, this study specificity is 45% and that ECF 26.8% of cows with ECF test negative results were in fact later confirmed to be pregnant. The ECF test PPV, NPV, specifity and sensivity are quite different according to days. It is a reality, ECF is present as early as 24 hours after mating until parturition. The problem is related to the tecnology of ECF test according to us.

In heifers embryonic mortality may account for 46 to 75% of pregnancy failures after AI (5). Serum progesterone has been used previously as an indicator of late embryonic mortality that occurs after days 14 post-breeding by the presence of extended inter-estrus intervals and sustained progesterone levels after day 24 (6). In this study progesterone levels were 2 ng/ml and embryo were not detected by ultrasound. According to results progesterone level is a good indicator of late embryonic mortality. However ECF test were positive in one of cows.

A survey performed by Sakonju et al. (9) the viability of the bovine embryo was monitored by measuring the early pregnancy factor. These reseachers reported, the measurement of EPF activity is useful for monitoring the viability of bovine embryos. While in the 20$^{th}$ days performed ultrasonographic examination 15 cows were pregnant, in the 30$^{th}$ and 45$^{th}$ days ultrasonografic examination repeated then 13 cows determined pregnant. In these two cows serum progesterone levels fell under 2 ng/ml. However, in 20$^{th}$ day this cows progesterone levels higher than 2 ng/ml. The embryonic death occurred in two cows. ECF test results at the day one cow was positive reaction. However, this cow was no pregnant by ultrasonographic examination. Our results are generally similiar the other researchers.

In conclusion, the results are shown that ECF test results are conflicting and changing according to days. ECF test is unreliable for pregnancy diagnosis and determining embryonic death in dairy cows so that ECF test cannot accurately identify the nonpregnant cow.

## References


1. *Adams C. S., Jardon P. W.*: Evaluation of the Early Conception Factor (ECF$^{tm}$) test in cows 3-7 days post-breeding. J. Dairy Sci. 1999, 82, Suppl. 1 (Abstract), 80-81.
2. *Broaddus B., De Vries A.*: A comparison of methods for Early Pregnancy Diagnosis, [in:] Proc 2$^{nd}$ Florida Dairy Road Show, Florida 2005, pp. 22-30.
3. *Cordoba M. C., Sartori R., Fricke P. M.*: Assessment of a commercially available early conception factor (ECF) test for determining pregnancy status of dairy cattle. J. Dairy Sci. 2001, 84, 1884-1889.
4. *Edmonson A. J., Lean I. J., Weaver L. D., Farver T., Webster G.*: A body condition scoring chart for Holstein dairy cows. J. Dairy Sci. 1989, 72, 68-78.
5. *Gandy B., Tucker W., Ryan P., Williams A., Tucker A., Moore A., Godfrey R., Willard S.*: Evaluation of the early conception factor (ECF$^{TM}$) test for the detection of nonpregnancy in dairy cattle. Theriogenology 2001, 56, 637-647.
6. *Humblot P.*: Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology 2001, 56, 1417-1433.
7. *Noakes D. E.*: Pregnancy and its diagnosis, [in:] Noakes D. E., Parkinson T. J., England G. C. W.: Arthur's Veterinary Reproduction and Obstetrics. Saunders, Elsevier Science Limited, China 2003, 69-118.
8. *Pennington J. A., Schultz L. H., Hoffman W. F.*: Comprasion of pregnancy diagnosis by milk progesterone on day 21 and dat 24 postbreeding: field study in dairy cattle. J. Dairy Sci. 1985, 68, 2740-2745.
9. *Sakonju I., Enomoto S., Kamimura S., Hamana K.*: Monitoring bovine embryo viability with early pregnancy factor. J. Vet. Med. Sci. 1993, 55, 271-274.
10. *Threlfall W. R., Bilderback G. M.*: Early conception factor (ECF) assay for nonconception determination in cattle. [in:] Proc. Soc. Theriogenology Ann. Conf., Baltimore 1998, pp. 157-159.


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