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Short communication: Test for nonpregnancy in dairy cows based on plasma progesterone concentrations before and after timed artificial insemination

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ABSTRACT

Timed artificial insemination (AI) programs have increased reproductive efficiency in dairy herds. A low timed AI pregnancy per AI is partially explained by cows that fail to respond optimally to the series of treatments that are designed to synchronize ovulation for AI. We hypothesized that testing cows for plasma progesterone concentrations during a timed AI protocol could be used as an early diagnostic test for nonpregnancy. Lactating Holstein cows (n = 160) in 2 confinement-style dairies were used. Cows were treated with Presynch Ovsynch 56 for timed AI. Concentrations of progesterone in plasma were measured at −3, 0, 7, and 25 d relative to timed AI. Progesterone data were analyzed and receiver operating characteristic curves were generated by using logistic regression. The area under the receiver operating curves for a progesterone test for nonpregnancy on d −3 ($PGF_{2\alpha}$), 0 (AI), 7, and 25 d relative to timed AI were 0.68, 0.52, 0.55, and 0.89, respectively. The cutpoints and sensitivity (respectively) for the progesterone test were 0.51 ng/mL (lower = nonpregnant) and 28.2% for the day of $PGF_{2\alpha}$, 0.43 ng/mL (greater = nonpregnant) and 17.9% for the day of AI, 1.82 ng/mL (lower = nonpregnant) and 23.1% for 7 d after AI, and 2.67 ng/mL (lower = nonpregnant) and 76.0% for 25 d after AI. The false positive rate was less than 5% for all tests. Analysis of a second data set from a published study gave approximately the same cutpoints and sensitivity. When both studies were combined, approximately 20% of nonpregnant cows could be identified with a single test that was done before or shortly after AI with a false positive rate of less than 5%. When 2 and 3 tests were applied sequentially, the sensitivity for identifying nonpregnant cows increased from 38.4 to 50.5%. The pregnancy per AI for those cows that met the established progesterone criteria was approximately 3 to 4 times greater than those that failed to meet the criteria. The conclusions were that cows destined to be nonpregnant after timed AI can be identified before or shortly after AI. Testing for nonpregnancy before or shortly after AI may have utility with respect to eliminating a nonproductive AI (cows identified before AI) or shortening the time to reinsemination (cows identified by 1 wk after AI).
Key words: progesterone, pregnancy, timed AI

Short Communication

Timed AI programs have increased reproductive efficiency in dairy herds (Thatcher and Santos, 2007; Wiltbank and Pursley, 2014). Refinements to existing programs have increased their effectiveness but pregnancies per AI (**P/AI**) to a single timed AI typically remain below 50% for most herds (Herlihy et al., 2012; Bisinotto et al., 2014). The low timed AI P/AI is partially explained by cows that fail to respond optimally to the series of treatments in the timed AI protocol that are designed to synchronize ovulation for AI (Pursley and Martins, 2011; Wiltbank et al., 2011). For example, the cohort of treated cows immediately preceding AI includes cows that (1) do not have a corpus luteum (**CL**) when $PGF_{2\alpha}$ is administered; (2) fail to undergo complete luteolysis after $PGF_{2\alpha}$; or (3) fail to ovulate after GnRH and timed AI. All 3 scenarios are associated with low fertility to timed AI (Pursley and Martins, 2011; Wiltbank et al., 2011). In a previous study (Escalante et al., 2013), we measured plasma progesterone concentrations in cows that underwent ovulation synchronization and timed AI. We then categorized cows based on changes in plasma progesterone into those that responded correctly to treatment and nonresponding cows. We found that P/AI was greater than 50% for cows that responded correctly to the treatments, whereas the P/AI for nonresponding cows was less than 20%. Based on these results and those of others (Ayres et al., 2013), we hypothesized that testing cows for plasma progesterone concentrations during a timed AI protocol could be used as an early diagnostic test for nonpregnancy. If an appropriate cutpoint for plasma progesterone concentration can be determined then it is theoretically possible to identify

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cows before or shortly after AI that are not pregnant. The nonpregnancy test before or shortly after AI would eliminate the unproductive days to pregnancy diagnosis (typically done 5 to 6 wk after AI) and create an opportunity for resynchronization within 1 to 2 wk after the initially planned timed AI.

Lactating Holstein cows (n = 160) in 2 confinement-style dairies (University of Missouri, Foremost Dairy, Midway, MO, and Heartland Dairy, La Belle, MO) were used. Cows were treated with Presynch Ovsynch 56 ($PGF_{2\alpha}$, 14 d, $PGF_{2\alpha}$, 14 d, GnRH, 7 d, $PGF_{2\alpha}$, 56 h, GnRH, 16 h, timed AI) so that first timed AI was 70 to 76 d postpartum. The $PGF_{2\alpha}$ was Lutalyse (5 mL; 25 mg; Zoetis, Florham Park, NJ). The GnRH was Factrel (gonadorelin hydrochloride; 2 mL; 100 μg; Zoetis). All inseminations were performed at timed AI. The cows were diagnosed for pregnancy by using a blood test for pregnancy associated glycoproteins (PAG; Idexx Bovine Pregnancy Test; Idexx Laboratories, Westbrook, MA) at 25 d after AI and then by using ultrasound by the herd veterinarian on 38 d after AI (Heartland Dairy) or by a theriogenologist from the University of Missouri College of Veterinary Medicine at 32 d after AI (Foremost Dairy). Initially, 177 cows were assigned to the trial, but 17 cows had a PAG result that did not agree with the ultrasound result and were excluded from the analysis so that 160 cows were used.

Blood samples were collected from the coccygeal vein into vacutainer tubes containing EDTA. Samples were put on ice until they reached the laboratory where they were centrifuged at 1,500 × *g* for 15 min and the plasma was stored at −20°C until analysis. Plasma progesterone concentrations were measured at −3, 0, 7, and 25 d relative to timed AI using the MP Biomedical Double Antibody 125I Kit for progesterone (MP Biomedical, Santa Ana, CA). A complete validation for this assay kit was recently published (Pohler et al., 2016). The samples were analyzed in a single assay with an intra-assay coefficient of variation of 7.7%.

Progesterone data were analyzed and receiver operating characteristic (**ROC**) curves were generated by using logistic regression in SAS (PROC LOGISTIC, SAS ver. 9.4; SAS Institute Inc., Cary, NC). The event was the result of the pregnancy diagnosis. For the purpose of the analysis, plasma progesterone concentration was evaluated as a test for nonpregnancy. The sensitivity (true positive rate) was defined as the proportion of nonpregnant cows that were correctly identified as nonpregnant for a given progesterone concentration (cutpoint). The specificity (true negative rate) was defined as the proportion of pregnant cows that were correctly identified as pregnant for a given progesterone concentration; 1-specificity is the false positive rate (pregnant cows incorrectly diagnosed as nonpregnant). Progesterone concentration for each of the days was tested independently in the analysis (−3, 0, 7, and 25 d relative to timed AI). Herd was included in the statistical model but later removed when found not significant. The ROC curve plots themselves were created by using a macro as described in the SAS Knowledge Base (Sample 25018: Plot ROC curve with cutpoint labeling and optimal cutpoint analysis; http://support.sas.com/kb/25/018.html). Cutpoints were selected that had the greatest sensitivity (true positive rate) with a false positive rate of less than 5%.

We noted 82/160 (51.3%) cows that were pregnant at the time of diagnosis by ultrasound. The area under the ROC curve for a progesterone test for nonpregnancy on d −3 ($PGF_{2\alpha}$ treatment) was 0.68 (Figure 1A; $P < 0.003$). A cutpoint at 0.51 ng/mL of progesterone on the day of $PGF_{2\alpha}$ had a sensitivity of 28.2% with a false positive rate of 3.7%, meaning that 28.2% of all nonpregnant cows would be correctly diagnosed (cows with ≤0.51 ng/mL diagnosed as not pregnant; Figure 1A). The same cutpoint would incorrectly diagnose 3.7% of pregnant cows as nonpregnant (false positive). The area under the ROC curve for a progesterone test for nonpregnancy on d 0 (day of AI) was 0.52 ($P < 0.05$; Figure 1B). A cutpoint of 0.43 ng/mL had a sensitivity of 17.9% with a false positive rate of 3.7%. The area under the ROC curve for a progesterone test for nonpregnancy on d 7 (7 d after AI) was 0.55 ($P > 0.10$; Figure 1C) and was not different from chance (area = 0.50). A cutpoint of 1.82 ng/mL had a sensitivity of 23.1% with a false positive rate of 4.9%. The area under the ROC curve for a progesterone test for nonpregnancy on d 25 after AI was 0.89 ($P < 0.001$; Figure 1D). A cutpoint of 2.67 ng/mL had a sensitivity of 76.0% with a false positive rate of 0%.

Progesterone concentrations immediately before AI are associated with improved fertility (Pursley and Martins, 2011; Wiltbank et al., 2011, 2014), and the favorable ROC curve area for the day of $PGF_{2\alpha}$ treatment (0.68) supports this observation. The cutpoint established for the day of timed AI (0.43 ng/mL) was similar to the 0.3 to 0.5 ng/mL range proposed by Wiltbank et al. (2014) as detrimental to fertility. Wiltbank et al. (2015) reported that administering a second $PGF_{2\alpha}$ treatment 24 h after the first decreased the percentage of cows with progesterone greater than 0.5 ng/mL from 17% (approximately equal to what we observed in our study) to 2.4%. The second $PGF_{2\alpha}$ treatment increased P/AI in the Wiltbank et al. (2015) study as would be expected based on the ROC curve that we present. A cutpoint of approximately 1.5 ng/mL was established 7 d after AI. This appears to be a suitable cutpoint for identifying cows that did not ovulate within 1 to 2 d after GnRH treatment. A non-