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Short communication: Field evaluation of a pregnancy confirmation test using milk samples in dairy cows

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ABSTRACT

The objective was to validate the performance under field conditions of a novel commercially available ELISA for confirmation of pregnancy using measurement of pregnancy-associated glycoproteins in milk samples from dairy cows. The target population was cows previously diagnosed pregnant by veterinary examination and ≥60 d of gestation. On 8 farms milking Holstein cows, milk samples were collected during routine Dairy Herd Improvement testing and shipped overnight to the Dairy Herd Improvement laboratory where the milk pregnancy test was performed. On the same day that milk samples were collected, transrectal palpation was performed by a veterinarian to confirm pregnancy status. Data were available from 683 cows, of which 661 were pregnant and 22 were not pregnant based on veterinary diagnosis, which was taken as the reference test. Based on the manufacturer's interpretive guidelines, 3.8% of test results were classified as "recheck," between the cut-points for classification of pregnant and nonpregnant and were not used in the analysis. The milk pregnancy test performance (and 95% confidence intervals) for confirmation of pregnancy was sensitivity of 99.2% (98.2 to 99.7%) and specificity of 95.5% (78.2 to 99.2%). Given a prevalence of 97% pregnant cows in the sample, the positive predictive value of the milk test was 99.8% (99.1 to 99.96%) and the negative predictive value was 80.8% (61.3 to 90.9%). When used to confirm pregnancy status or detect fetal losses at ≥60 d gestation in cows previously diagnosed pregnant, the recommended action for cows with a milk pregnancy-associated glycoprotein test result of not pregnant is veterinary reexamination of the animal to confirm the presence or absence of a viable fetus before reinsemination or administration of prostaglandin.

Key words: pregnancy diagnosis, pregnancy confirmation, pregnancy-associated glycoprotein

Short Communication

Timely pregnancy is economically important for dairy herds and, consequently, timely and accurate pregnancy diagnosis is a key element of management of reproduction. Initial diagnosis of nonpregnancy is necessary so that cows can be reinseminated if not pregnant to a previous AI. Conversely, among cows correctly diagnosed pregnant, loss of pregnancy is common, especially between early diagnosis of pregnancy at 28 to 30 d of gestation and 56 to 60 d of gestation, during which time approximately 15% of pregnancies may be lost (Santos et al., 2004), although these losses are more likely in the earlier part of this interval. From approximately 60 d of gestation to term, the rate of fetal loss is reduced but 2 to 4% of pregnancies are typically lost (Santos et al., 2004). Recent data from DHI records in the United States indicate 1.3% abortion from 151 d of gestation to term (Norman et al., 2012).

Pregnancy-associated glycoproteins (**PAG**) are a group of >20 proteins produced by binucleate cells in the bovine placenta, with increases detectable in plasma in pregnant cows starting at approximately 24 d of gestation, and are diagnostically useful at 28 to 30 d after AI (Romano and Larson, 2010; Thompson et al., 2010; Fricke and Giordano, 2011). The concentrations of various PAG vary with the stage of gestation and decline when fetal loss occurs (Giordano et al., 2012). Recently, a test has been developed to measure PAG in milk, in addition to existing validated tests for PAG in plasma or serum. The objective was to validate the performance of a novel commercially available ELISA for confirmation of pregnancy using milk samples from dairy cows.

The study population was a convenience sample of 8 dairy herds milking Holstein cows in southwestern Ontario, Canada. The herds were chosen based on using DHI service (CanWest DHI, Guelph, ON, Canada), having accurate computerized reproduction records, exclusively using AI, and willingness to participate when asked. The herds were also selected to provide a variety of brands and types of milking equipment (7 freestall barns with parlors and 1 tiestall barn) and milk meters. The herds had between 97 and 496 cows

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being milked (mean ± SD: 266 ± 168), with milk production between 33 and 42 kg/cow per day (37 ± 11 kg/cow per day) on the day of sampling. Each herd was visited once in May or June 2012. On the day of the visit, a routine DHI milk-recording test was conducted. A composite milk sample was collected from each cow, preserved with bronopol, and shipped overnight to the DHI laboratory (CanWest DHI). The order of milking (i.e., the sequence of cows milked at each stall in the parlor or with each milking unit) was recorded. At the laboratory, samples from cows ≥60 DIM and ≥60 d pregnant, or not pregnant but ≥60 d since the last AI based on the herd's records on the day of sampling were analyzed using a commercially available 96-well microplate ELISA for detection of PAG in milk (Idexx Laboratories Inc., Westbrook, ME). Technicians collecting samples and performing the ELISA were blinded to the cows' pregnancy status. The ELISA test was performed by a trained technician according to manufacturer's instructions. A microtiter plate was coated with anti-PAG antibody. After incubation in the well, captured PAG was detected with a PAG-specific antibody and a horseradish peroxidase conjugate. Unbound conjugate was washed away, and colorimetric substrate was added to the wells. Color development was proportional to the amount of PAG in the sample and was measured using a spectrophotometer. Results were calculated from the optical density (**OD**) of the sample (S) minus the OD of the negative control (N) at 450 nm (with both values corrected by subtraction of the reference wavelength OD), which resulted in an S − N value. Each microplate included negative and positive control and reference samples. Based on the manufacturer's guidelines, samples with S − N <0.100 were classified as not pregnant, S − N ≥0.100 but <0.250 as "recheck," and S − N ≥0.250 as pregnant. Because residual PAG from the previous pregnancy is detectable after calving, the manufacturer's specification is to use the test only in cows >60 DIM. Based on the objective of the present study and the intended application of the test, assuming submission of samples through routine monthly DHI testing, samples from cows previously diagnosed pregnant and ≥60 d of gestation were analyzed. Additional samples were taken from cows expected to be nonpregnant (i.e., "do not breed" cows that the farm managers had elected not to inseminate but that were still in the herd lactating) and that were also ≥60 d since the last insemination; these were included to increase the precision of the estimate of the specificity of the test. On 7 farms, essentially all cows meeting the study-inclusion criteria were examined by an experienced veterinarian to confirm pregnancy status by transrectal palpation [<2% of cows per farm that met the inclusion criteria were not examined if the cow could not be positively identified (e.g., missing ear tags) or could not be caught]. On one farm, milking 464 cows, not all eligible cows could be physically processed sufficiently quickly through the handling facility (management rail), so a random sample of 219 cows meeting the inclusion criteria was used. Pregnancy was confirmed if placentomes or the fetus was palpated. Veterinarians were aware of the cow's recorded pregnancy status and stage (days of gestation).

The sample size was estimated based on establishment of noninferiority (one-sided test, 99.5% statistical confidence) of the milk test to within 2 to 2.5% points of veterinary diagnosis (assuming 2% discordant results between the milk test and palpation), resulting in sample sizes of 794 or 516 cows, respectively (Abramson, 2011).

Veterinary diagnosis by rectal palpation was taken as the reference test (gold standard) to which the milk pregnancy test was compared. Performance of the latter was assessed by calculating the sensitivity (proportion of pregnant cows classified as pregnant), specificity (proportion of nonpregnant cows classified nonpregnant), positive predictive value (proportion of milk pregnancy test results of "pregnant" for which the cow was pregnant) and negative predictive value (proportion of milk pregnancy test results of "open" for which the cow was not pregnant). These measures and their 95% confidence intervals (Zou's method) were calculated using WinPepi version 11.22 (Abramson, 2011; http://www.brixtonhealth.com/pepi4windows.html).

Milk pregnancy test results and concurrent veterinary pregnancy diagnosis were available from 710 cows that met the study inclusion criteria, of which 27 (3.8%) had a result of "recheck," leaving 683 cows in the final analysis. Of these, 665 had been previously diagnosed pregnant and 18 were recorded in the farm records as not pregnant; all were ≥60 d since the last AI. Among 661 cows actually diagnosed pregnant on the day of sampling and examination, the mean (±SD) stage of gestation was 140 ± 49 d (range from 60 to 230 d) and among 22 nonpregnant cows, the mean interval from the last AI was 153 ± 83 d (range from 61 to 341 d). The data and test performance are summarized in Table 1. Sensitivity (classification of pregnant cows as pregnant) was 99.2% and specificity (classification of open cows as open) was 95.5%. The kappa statistic was 0.87, indicating overall excellent agreement beyond chance between the diagnostic methods. With 661 of 683 cows pregnant, the positive predictive value (probability that a cow with a milk result of "pregnant" was pregnant) was 99.8% and the negative predictive value (probability that a cow with a milk result of "open" was open) was 80.8%.