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Technical note: Validation of a chemical pregnancy test in dairy cows that uses whole blood, shortened incubation times, and visual readout

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ABSTRACT

Chemical pregnancy testing is an alternative to traditional methods of pregnancy diagnosis (either manual palpation or ultrasound) in postpartum dairy cows and heifers. The objective was to validate a chemical pregnancy test that confers the advantages of using whole blood, rapid incubation times, and visual readout. Blood and milk samples were collected from Holstein dairy cows [n = 320; 162 ± 62 (mean ± SD) d in milk] on a confinement farm in northeast Missouri at 28 d after artificial insemination (AI). The samples were assayed for pregnancy-associated glycoproteins (PAG) by using a rapid visual test as well as traditional plasma- and milk-based tests. Transrectal ultrasonography diagnosis for pregnancy at 35 to 38 d after AI was the reference (gold) standard for all PAG tests. One hundred fifty-nine cows were diagnosed as pregnant by the reference standard (pregnancies per AI = 49.7%). The tests were ELISA and either optical density (OD; measured with a microtiter plate reader; plasma, milk, and rapid visual tests) or visual readout (rapid visual test) were used to diagnose pregnancy. When OD was used, the percentage of pregnant cows classified correctly (sensitivity) for the plasma, milk, and rapid visual tests were 97 ± 1, 96 ± 2, and 95 ± 1% (±SE), respectively. The sensitivity of the rapid visual test when assessed visually was 98 ± 1%. The specificity (proportion of nonpregnant cows classified correctly) for the plasma, milk, and rapid visual was 94 ± 2%, 94 ± 2%, and 93 ± 2% when an OD was used. When read visually, the specificity of the rapid visual test was lesser (85 ± 3%) because some cows with faint visual signals yielded false positive diagnosis. The overall accuracy (proportion of pregnant and nonpregnant cows diagnosed correctly) was similar for all tests (plasma, milk, rapid visual OD, and rapid visual; 96 ± 1, 95 ± 1, 94 ± 1, and 92 ± 2%, respectively). In a second experiment, lactating Holstein cows (n = 291) from 4 commercial confinement dairy farms in western Kentucky were tested 25 to 95 d after AI using the rapid visual test. The OD of the rapid visual test followed the known profile for PAG in circulation during the first trimester of pregnancy. The conclusion is that the rapid visual test has equal sensitivity and accuracy as existing PAG tests. A slightly lower specificity was found when the rapid visual test was read visually.
Key words: pregnancy diagnosis, pregnancy-associated glycoprotein, ELISA

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Pregnancy diagnosis within 4 to 6 wk after AI in postpartum dairy cows is critical for identifying nonpregnant cows eligible for rebreeding so that time from calving to conception (days open) is less (Silva et al., 2009; Giordano et al., 2013). Traditional methods of pregnancy diagnosis such as ultrasonography or manual palpation are typically performed 32 or more days after AI (Fricke, 2002). Earlier tests are based on pregnancy-associated glycoproteins (**PAG**) in the circulation that are detected as early as 25 d after AI in cattle (Green et al., 2005, 2009; Wallace et al., 2015). The PAG can be detected in either plasma, serum, or milk (Silva et al., 2007; LeBlanc, 2013; Lawson et al., 2014). The accuracies of commercial PAG tests for plasma, serum, or milk range from 89 to 96% (Silva et al., 2007; Karen et al., 2015; Ricci et al., 2015). The accuracy of PAG tests makes them suitable alternatives to traditional methods of pregnancy diagnosis (palpation or ultrasound). There are limitations, however, to their use. Plasma or serum-based tests require the collection of a blood sample and subsequent centrifugation. The centrifugation step requires equipment that is typically not available on farm. Milk testing does not require centrifugation, but the initial assay step requires a thermally controlled platform shaker for the ELISA plate. Shaking is not required for assays performed with plasma or serum, but an incubator is needed. Regardless of whether plas-

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ma, serum, or milk is used, a microtiter plate reader is required to measure the optical density (**OD**) for individual wells in the ELISA plate. Given the need for specialized equipment, PAG testing is most likely done in a veterinary clinic or other centrally located laboratory. A final impediment to the on-farm test is the time from sample collection to pregnancy test result. Traditional tests have a series of incubations with antibody and reagent solutions that can require from 2 to 3 h or as long as overnight to complete the test. In many herds, treatments are administered to nonpregnant cows after pregnancy diagnosis, usually before they are released from animal restraint (headlocks, squeeze chute, or palpation rail). The length of time from sampling to test completion is too long if cows are to remain restrained or kept in separate pens. A lesser time from sampling to test result could improve on-farm utility of a PAG test. We tested a rapid visual PAG test (Rapid Visual Pregnancy Test; Idexx, Westbrook, ME) that addresses some of the limitations listed above. Specifically, whole blood (EDTA) can be used in addition to plasma (EDTA) or serum, total test time is reduced to approximately 30 min, and the plate can be read visually. The objective of this experiment was to validate the rapid visual test performed 28 d after AI by using transrectal ultrasound on d 35 to 38 as the reference standard. We also compared the results obtained from the rapid visual test with those obtained using traditional PAG tests based on plasma or milk. Finally, the rapid visual test was used to assay a series of samples collected during the first trimester of pregnancy (d 25 to 95).

The validation experiment was performed at a confinement dairy farm in northeast Missouri by using 2 cohorts of lactating Holstein cows (n = 320) in January 2016. The cows were 162 ± 62 (mean ± SD) DIM on the day of blood and milk sample collection. Both primiparous (n = 95) and multiparous (n = 225) cows were used (mean ± SD = 2.3 ± 1.2 calvings). Cows were housed in a standard free-stall, milked 3 times daily, and fed a TMR. Cows were treated with a Presynch Ovsynch_56 protocol [$PGF_{2\alpha}$, 14 d, $PGF_{2\alpha}$, 14 d, GnRH, 7 d, $PGF_{2\alpha}$, 56 h, GnRH, 16 h, timed AI ($PGF_{2\alpha}$ = Lutalyse, 5 mL i.m., 25 mg, Zoetis Animal Health, Florham Park, NJ; GnRH = Fertagyl, 2 mL i.m., 100 μg, Intervet, Milan, Italy)] so that first timed AI was 70 to 76 d postpartum. Cows submitted to a timed AI (n = 114) or resynchronization timed AI (n = 206) were enrolled in the study. The resynchronization protocol began with a GnRH treatment 32 d after timed AI and concluded with a resynchronized timed AI on d 42 (GnRH, 6 d, ultrasound pregnancy diagnosis, 1 d, and then for nonpregnant cows, $PGF_{2\alpha}$, 56 h, GnRH, 16 h, timed AI). The number of inseminations at the time of pregnancy diagnosis averaged 2.3 ± 1.4 (mean ± SD).

Blood and milk samples were collected 28 d after timed AI. Cows eligible for pregnancy diagnosis from the herd were identified by radio frequency emitting ear tags upon exit from the parlor and directed to a standard palpation rail that was used to restrain cows during sampling. Blood and milk samples were collected while cows stood in the palpation rail. Blood was collected by coccygeal venipuncture into a Monoject tube containing 100 μL of a 15% solution of EDTA (K_3; Covidien, Minneapolis, MN). Milk was collected into a 20-mL disposable polyethylene scintillation vial (Fisherbrand, Pittsburgh, PA). Blood and milk sample tubes were placed on crushed ice immediately after collection, transported back to the laboratory within 8 h of sampling, and stored overnight at 4°C. The following morning, blood tubes were inverted to mix and approximately 2 mL of whole blood was transferred to a 12 × 75 mm borosilicate glass tube for subsequent use in the rapid visual test. The remaining blood was centrifuged at 1,500 × *g* for 15 min at 4°C. Plasma for use in the conventional test was aspirated into a 12 × 75 mm polypropylene tube. Milk was mixed thoroughly by inversion before use.

All assays were performed according to the manufacturer's instructions. The purpose of this study was to validate the performance of the rapid visual test (Idexx Rapid Visual Pregnancy Test) relative to transrectal ultrasonography as the reference (gold) standard for pregnancy diagnosis. A conventional test that can use either plasma or serum (Idexx Bovine Pregnancy Test) and a milk-based test (Idexx Milk Pregnancy Test) were also compared with the reference standard as well as to the rapid visual test.

The rapid visual test is a sandwich-style ELISA. It has a similar format to conventional (plasma or serum) and milk tests but some steps are combined and incubation times are shorter. Although performance of the test with whole blood (EDTA) is presented, plasma (EDTA) or serum can also be tested. The manufacturer provides 96-well assay plates comprised of twelve 8-well assay strips. For the rapid visual test, 100 μL of whole blood and 3 drops (approximately 100 μL) of reagent 1 detector solution were added to individual wells of the PAG antibody-coated ELISA plate. The plate was covered, tapped gently to mix, and incubated for 7 min at room temperature (23 to 26°C). After 7 min, the solution in the wells was removed by inversion and then the wells were washed thrice with distilled water. For washing, individual wells were gently filled until overflowing by using a 500-mL wash bottle (no. 414004–227; VWR, Radnor, PA) and a flow rate of approximately