ABSTRACT

Results from milk progesterone assays can provide ovarian information, making it possible to monitor the reproductive status of the cow. Rapid, simple, and accurate on-farm milk progesterone tests are commercially available. Procedures for performing milk progesterone tests are specific for each test; however, principles of testing are similar. On-farm tests are designed to determine the relative progesterone concentration ("high" or "low") rather than obtaining a precise concentration. Evaluation of results is based on either a color or agglutination reaction with comparison to known standards. Potential uses of progesterone testing in a reproductive management program are: 1) identifying errors in detection of estrus, 2) predicting time of estrus, 3) assisting in pregnancy diagnosis, 4) differentiating types of ovarian cysts, and 5) evaluating response to endocrine therapy.

INTRODUCTION

Laing and Heap (26) first demonstrated that concentrations of progesterone in the milk of cows reflected the stage of the estrous cycle as assessed by palpation of the ovary per rectum. Progesterone concentrations in milk were later shown to parallel those in plasma (20, 22). Numerous workers have reported the usefulness of measuring milk progesterone for monitoring the cow's reproductive status (3, 13, 18, 24, 26). As a method of pregnancy diagnosis, milk progesterone concentration at d 21 to 24 postinsemination is 67 to 88% accurate for diagnosing cows subsequently determined to be pregnant and 91 to 100% accurate for cows determined to be not pregnant (2, 8, 9, 13, 18, 21, 29, 38, 39, 53).

Until recently, determination of progesterone in a large number of samples was based on radioimmunoassay (RIA) and confined to specialized laboratories. The potential advantages of enzyme-linked immunosorbent assays (ELISA) over RIA have been discussed previously in terms of general accessibility, low cost, and innocuous nature of reagents employed (7, 27, 46). Furthermore, ELISA performed on-farm eliminates the shipping, processing, and response time of 2 to 10 d (9) and involves no sample preparation, i.e., an assay carried out on whole milk without prior extraction of steroid. Development of progesterone ELISA (11, 12, 30, 32, 33, 43, 44, 50, 54) has removed many of the constraints imposed by RIA. Correlations of ELISA with a previously validated RIA has been reported where both methods were performed in laboratory .93 and .90 (11, 51). Where ELISA was performed on-farm and RIA in the laboratory, correlation was .79 (36). Milk progesterone ELISA methods will likely replace RIA methods for most laboratory and on-farm applications.

MILK PROGESTERONE TEST PRINCIPLES

Enzyme-Linked Immunosorbent Assay

The first application of ELISA for the assay of small molecules was in the assay of progesterone (14). Procedures for performing ELISA milk progesterone assays are specific for each company producing a test; however, the principles of the assay are similar. Methods for ELISA are based on the same principles as for RIA. The major difference between ELISA and RIA is use of an enzyme to label progesterone rather than a radioactive isotope. The ELISA tests are currently designed to determine if
progesterone concentrations are "low" or "high" rather than providing a precise concentration as RIA procedures do. The ELISA methods produce a color reaction (yellow, blue, or pink) determined by the conjugate-substrate system employed.

An ELISA milk progesterone assay is diagrammatically shown in Figure 1. The assay utilizes the principles of competitive absorption of milk progesterone to an antibody specific for progesterone. Progesterone antibodies are coated to a plastic tube. Tests commercially available use plastic test tubes, microtiter plates and strips, plastic and fiber dip sticks, and fiber discs (Table 1). Regardless of antibody attachment site, assay principles are similar. When milk is added to the system, progesterone attaches to specific progesterone antibody binding sites (Figure 1, step 1). For most tests, immediately following milk addition, a progesterone-enzyme conjugate is added (step 2). Three progesterone-enzyme conjugates have been reported, alkaline phosphatase (45), β-galactosidase (19, 35, 43, 44), and horseradish peroxidase (1, 11, 30, 32, 50, 52). Progesterone-enzyme conjugate attaches to unbound antibody sites; therefore, the greater the progesterone concentration, the lower the binding of conjugate to antibody. Following a brief incubation (1 to 15 min) tubes are emptied and rinsed with water. The next step is substrate addition (Figure 1, step 3). Degradation of substrate, designed specifically for the conjugate system employed, is inversely proportional to concentration of progesterone. The final step is addition of developer (chromogen) which reacts with modified substrate to produce a color reaction (substrate and chromogen are combined in some assays). The color reaction is evaluated after a brief incubation of 1 to 5 min depending upon specific test being used. Amount of color development is inversely proportional to the concentration of progesterone in milk. For example, a sample from

![Figure 1. General principles of an on-farm milk progesterone enzyme-linked immunosorbert assay.](image-url)
<table>
<thead>
<tr>
<th>Item</th>
<th>Accufirm&lt;sup&gt;1&lt;/sup&gt;</th>
<th>B.E.S.T.&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Calfcheck&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Enzgno&lt;sup&gt;4&lt;/sup&gt;</th>
<th>EstruCHEK&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Open Alert&lt;sup&gt;7&lt;/sup&gt;</th>
<th>OvuSure Rapid Tubc&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Target&lt;sup&gt;6&lt;/sup&gt;</th>
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<td>6</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Type of sample</td>
<td>Strippings</td>
<td>Foremilk</td>
<td>Strippings</td>
<td>Strippings</td>
<td>Foremilk</td>
<td>Foremilk</td>
<td>Foremilk</td>
<td>Foremilk</td>
</tr>
<tr>
<td>Site of antibody</td>
<td>Plastic tube</td>
<td>None</td>
<td>Dip stick (plastic)</td>
<td>Microtiter plate</td>
<td>Microtiter strip</td>
<td>Dip stick (fiber)</td>
<td>Plastic tube</td>
<td>Porous disc</td>
</tr>
<tr>
<td>conjugate system</td>
<td>Horseradish peroxidase</td>
<td>None</td>
<td>Horseradish peroxidase</td>
<td>Alkaline phosphatase</td>
<td>Horseradish peroxidase</td>
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</tr>
<tr>
<td>Time required (min)</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>40</td>
<td>22</td>
<td>10</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>How results</td>
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<td>Texture of milk</td>
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<td>Solution color</td>
<td>Pad color</td>
<td>Solution color</td>
<td>Disc color</td>
<td></td>
</tr>
<tr>
<td>interpreted</td>
<td>reader film</td>
<td>(blue)</td>
<td>(pink)</td>
<td>(blue)</td>
<td>(blue)</td>
<td>(blue)</td>
<td>(blue)</td>
<td></td>
</tr>
<tr>
<td>Max. test</td>
<td>24 small</td>
<td>15</td>
<td>23 small</td>
<td>96</td>
<td>36</td>
<td>12 small</td>
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<td>per kit</td>
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<td></td>
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<td>60 large</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>1</sup> Kamar Inc., Steamboat Springs, CO.
<sup>2</sup> Gist-Brocades USA Inc., Charlotte, NC.
<sup>3</sup> West Agro Inc., Kansas City, MO.
<sup>4</sup> Noctech Ltd., Dublin, Ireland.
<sup>5</sup> Hoechst-Roussel Agri-Vet, Summerville, NJ.
<sup>6</sup> Synbiotics Corp., San Diego, CA.
<sup>7</sup> An-Tech International, Phoenix, AZ.
<sup>8</sup> Elanco Products, Indianapolis, IN.
<sup>9</sup> BioMetallics Inc., Princeton, NJ.
<sup>10</sup> Norden Laboratories, Lincoln, NE.
a cow in mid estrous cycle or a pregnant cow will result in a slight color change or remain clear. In contrast, a sample from a cow in estrus, which has low progesterone, will yield a dark or intense color change.

Classification of the color reaction is a major limitation of ELISA tests. To overcome this problem a sample or samples of known progesterone concentration are analyzed simultaneously with desired milk samples. Most test kits include two standards for comparison: one for estrus ("low") and one for pregnancy ("high"). Other tests utilize a single known progesterone standard for comparison. When using the single standard system, concentrations associated with estrus result in a darker than standard reaction, whereas concentrations above the standard are associated with the nonestrual portion of the cycle or pregnancy. Assays from such cows produce a lighter color development than known standard.

**Latex Agglutination Test**

A procedure utilizing progesterone monoclonal antibodies and latex beads coated with progesterone has been developed to quantify rapidly the concentration of progesterone in milk (Table 1). Equal amounts of milk, antibody, and progesterone-coated latex beads are mixed together and applied to a reaction slide. As the mixture diffuses across the slide in a narrow channel, the latex beads and solutions interact with each other to provide a thin milk film. Milk containing a high concentration of progesterone results in progesterone antibody binding with no agglutination and appearing as a smooth milk film in chamber. In contrast, low concentrations of progesterone cause progesterone-coated latex beads to cluster or agglutinate, resulting in a grainy film in the slide chamber. This test does not use a standard for comparison, since the final indicator is a smooth vs. grainy appearance of the milk film.

**ON-FARM MILK PROGESTERONE TESTS**

Rapid on-farm milk progesterone tests are available. Important characteristics of 8 commercially available milk progesterone kits are outlined in Table 1. Most tests require either foremilk or last milk stripings. It is important that the type of sample specified for a particular test be used. Hoffman and Hamburger (23) showed that method of sampling and fat percentage of milk affected progesterone concentration in milk. They reported that, as a general rule, progesterone concentration of milk increased by 3 ng/ml for each percentage unit increase in milk fat. The control or standard that is tested simultaneously for comparison along with other principles of the test are specific for the type of sample being analyzed.

Test results can be obtained within 2 to 40 min depending on the specific test used with cost per sample being $2 to $7, depending primarily on number of samples analyzed simultaneously. The cost effectiveness of milk progesterone testing needs to be investigated and weighed against the possible increase in reproductive efficiency. Characteristics of each of the currently available kits are as follows.

**Accufirm™ and RPT™**

Accufirm (Kamar Inc., Steamboat Springs, CO) and RPT (Gist-Brocades Inc., Charlotte, NC) kits are identical having the same manufacturer (Immucell Corp., Portland, ME). A maximum of seven samples can be assayed simultaneously with a single standard for colorimetric comparison. Sample classification can be performed visually or by use of electronic scanner that uses the difference in optical density at 450 nm to distinguish color intensities to classify progesterone concentrations as high or low.

**B.E.S.T.™**

The B.E.S.T. test (West Agro Inc., Kansas City, MO) is the only non-ELISA milk progesterone test commercially available. It is based on a reaction between latex beads coated with progesterone molecules and antibodies, which have a specific attraction to progesterone as previously described.

**Calfcheck™**

Calfcheck (Noctech Ltd., Dublin, Ireland) was introduced in 1983 (31) utilizing an eight-well microtiter strip and was modified in 1987 to a dip stick format. The monoclonal antibody-coated dip stick has eight sides for maximum surface area.
Enzygnost™

The enzygnost kit (Hoechst-Roussel Agri-Vet Comp., Summerville, NJ) contains a 96-well microtiter plate, arranged as six 8 × 2-well strips in a frame. Procedures unique to this ELISA are addition of amplifier to enhance sensitivity or intensity of color and a .3 M sulfuric acid stopping solution for maintenance of final reaction color (54). A series of standards (1, 5, 10, and 30 ng/ml) are included for laboratory quantitative analysis with a microtiter plate reader (16).

EstruCHEK™

EstruCHEK (Synbiotics Corp., San Diego, CA) utilizes a detachable microtiter well system arranged in three strips of 12 wells each. Wells can be individually detached with desired number (maximum of five samples) placed in bracketed holder for manipulation.

Open Alert™ and BoviPro21™

Open Alert and BoviPro21 (An-Tech International, Phoenix, AZ) employ a dip stick ELISA procedure. Each dip-stick contains two antibody-coated fiber pads. One pad contains a progesterone standard; therefore, each dip stick contains a reactive pad for desired sample and standard for comparison. Each test consists of two premeasured vials; one contains lyophilized conjugate and the other contains .5 ml of substrate-chromogen mixture. Test results are permanent, and sample identification can be recorded on each strip for convenient record-keeping.

OvuSure™

OvuSure (Elanco Products Co., Indianapolis, IN) has two separate kits; one utilizes microtiter wells and the other plastic tubes for site of antibody attachment. A maximum of five samples can be assayed simultaneously and compared with the standard for classification of samples.

Target™

The Target kit (BioMetallics Inc., Princeton, NJ) contains 20 monoclonal antibody-treated test cups. The procedure utilizes a test cup for each sample with an antibody-coated porous disc located in base of the cup. Capillary action forces reagents across the disc. A 3 ng/ml low standard produces a blue color on a disc for sample comparison. A colorless disc occurs at progesterone concentrations above 10 ng/ml, which is used as the high standard. Substrate and chromogen must be prepared prior to use.

APPLICATIONS OF ON-FARM MILK PROGESTERONE TESTS

On-farm milk progesterone tests are designed primarily for use by dairy producers and veterinarians for assessment of stage of the estrous cycle and determination of pregnancy or nonpregnancy. However, milk progesterone testing is only a management aid and, like all aids, should be used judiciously.

Identifying Errors in Detection of Estrus

Accurate detection of estrus is essential for successful use of AI. The failure to observe (4, 18, 42, 49) and to interpret correctly signs of estrus (18, 22, 40) results in significant economic losses (4, 10, 37). An important use of on-farm milk progesterone tests is verification of progesterone concentrations of cows with questionable signs of estrus. Low milk progesterone concentration alone is not a positive indicator of estrus; however, high milk progesterone reliably confirms the nonestrual condition, even in animals having behavioral symptoms of estrus. The proportion of cows not in or near estrus when inseminated varies from 0 to 60% among herds (40). It is recommended that an on-farm milk progesterone test be performed at milking after estrus is suspected for any of the following reasons: 1) standing estrus that does not have a 18 to 24-d interval from prior estrus, 2) detection of estrus solely by secondary signs or by mechanical detection aids, and 3) detection of estrus for cow previously diagnosed pregnant.

Prediction of Estrus

Timed insemination after a decline in milk progesterone concentration has been reported by Foulkes and coworkers (19). Of the 164 cows, 82 were bred 2 d after detecting the characteristic fall in milk progesterone concentration accompanying regression of the
corpus luteum and 82 served as controls being observed in estrus and inseminated during the same period. Of the 82 cows in progesterone-analyzed group, 80 were inseminated during the 30-d trial with 64% having high milk progesterone concentrations 24 d postinsemination. In the control group that was visually observed and inseminated at estrus, 58 of these 82 cows were inseminated during the 30-d trial with 67% having high milk progesterone concentrations 24 d postinsemination.

In a similar study designed to predict estrus using an ELISA progesterone test, two herds consisting of 250 (herd A) and 104 (herd B) cows were used (15). Milk samples were collected and analyzed for progesterone concentration 16 to 24 d (herd A) or 17 to 23 d (herd B) postinsemination. Following a decline in progesterone concentration a heat detector was applied. Cows were inseminated either after an observed estrus or after the heat detector was triggered. The proportion of estrus intervals between 18 and 24 d improved from 28 to 67% for herd A and 21 to 45% for herd B. Of cows with low progesterone on d 24, 66.4% were low on d 19 and 94% low on d 22. Furthermore, 82% of cows had low progesterone concentrations for more than 48 h before they were bred. Only 66% of the breedings occurred on d 3 and 4 after initial progesterone testing, thus fixed time insemination was considered to be unacceptable because of the possibility of reduced pregnancy rates. These results indicate that sampling on d 18, 20, 22, and 24 should predict estrus with a high degree of certainty, but sampling on d 19, 21, and 23 could be a practical alternative. Progesterone tests only accurately identify cows not in estrus.

A breeding program incorporating ELISA milk progesterone assays and prostaglandin treatment to circumvent detection of estrus has been reported (17). Once-a-week milk samples were obtained from all known nonpregnant, reproductively normal cows greater than 60 d postpartum for evaluation with ELISA of milk progesterone. Cows with high progesterone concentrations were administered prostaglandin F2α and bred AI 72 and 96 h following prostaglandin administration regardless of signs of estrus. Cows with low progesterone concentrations were reassayed 7 d later and administered prostaglandin and bred AI 72 and 96 h if high progesterone concentrations were obtained. Pregnancy rates were not different from prior pregnancy rates of the herd; however, benefit of the program was labor savings.

Identification of Open Cows

Accurate determination of pregnancy or more importantly, which cows are not pregnant is an essential part of good reproductive management. Historically, pregnancy in cattle has been determined by nonreturn to estrus or uterine palpation per rectum 35 d or more postinsemination. Accuracy of identifying pregnant animals is consistently lower than for nonpregnant animals, presumably due to embryonic mortality, inaccurate sampling, breeding during luteal phase, prolonged luteal lifespan, and luteal cysts. A high progesterone concentration 21 to 24 d postinsemination is only an indirect indication that conception has occurred, and close observation for signs of estrus should be continued.

Differentiation of Ovarian Cysts

Ovarian cysts are an important infertility problem in dairy cattle (24, 25). Furthermore, ovarian cysts are often classified into two categories: 1) follicular cysts and 2) luteal cysts (6, 41). It is often difficult to differentiate ovarian cysts into follicular and luteal by palpation of the ovary per rectum (22, 47). Variation in the amount of cyst wall luteinization makes it difficult to distinguish between follicular and luteal cyst by palpation (28, 35, 47). However, once the presence of an ovarian cyst has been determined by palpation, differentiation can be made on the basis of progesterone concentration (28, 35). Cystic cows having a low progesterone concentration are considered to have follicular cysts, and those with high concentrations are classified as having luteal cysts. For practical purposes, differential diagnosis of ovarian cysts is important in selection of endocrine therapy (28).

Evaluating Response to Endocrine Therapy

Milk progesterone assay results can facilitate evaluation of any endocrine treatment which
TABLE 2. Accuracy of enzyme-linked immunosorbent assay (ELISA) milk progesterone results for diagnosis of pregnancy from samples collected 20 to 24 d postinsemination.

<table>
<thead>
<tr>
<th>Study</th>
<th>(n)</th>
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<tbody>
<tr>
<td>11</td>
<td>115</td>
</tr>
<tr>
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<td>152</td>
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<tr>
<td>34</td>
<td>268</td>
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<tr>
<td>36</td>
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<tr>
<td>54</td>
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<table>
<thead>
<tr>
<th>Classification of postinsemination sample</th>
<th>Pregnant (%)</th>
<th>Not pregnant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>67.2–87.5</td>
<td>95–98.3</td>
</tr>
<tr>
<td>30</td>
<td>96</td>
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</tr>
<tr>
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<td>100</td>
</tr>
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<td>69.7</td>
<td>93.9</td>
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<td>43</td>
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<td>71</td>
<td>81</td>
</tr>
<tr>
<td>54</td>
<td>92.1</td>
<td>100</td>
</tr>
</tbody>
</table>

affects ovarian status. Milk progesterone testing 10 d after administration of gonadotropin-releasing hormone (GnRH) to monitor effective luteinization of follicular cyst has been reported (35). Of 104 cows with follicular cysts, as defined by ELISA of milk progesterone, 73 (70%) responded to treatment with GnRH, whereas palpation of ovaries 10 d after GnRH treatment was 30% accurate in judgement of follicular cyst luteinization. Therefore, progesterone testing 10 d after administration of GnRH could accurately determine if retreatment is needed.

Prostaglandin compounds are extremely effective luteolytic agents. However, unobserved estrus following prostaglandin administration is a common occurrence. Response to prostaglandin is dependent upon the presence of a functional corpus luteum. Therefore, milk progesterone testing prior to and 3 d after administration of prostaglandin (5) can accurately assess if administration is justified (high concentration prior to administration) and if luteolysis has occurred (low concentration on d 3).

CONCLUSION

On-farm milk progesterone testing can be a useful tool to both dairy producers and veterinarians for assessment of ovarian status. On-farm tests are currently designed to determine the relative progesterone concentration rather than providing quantitative results. Many tests have been developed and are commercially available (Table 1). The type of milk sample recommended, foremilk, composite, or stripplings and utilization of blood serum, is different for different tests. Studies in our laboratory (unpublished) and elsewhere (48) revealed that results obtained from various commercial milk progesterone kits are similar; however, all possible comparisons have not been performed. Additionally, research is needed to determine if simple, quick, and accurate tests for determining relative milk progesterone concentrations will improve reproductive efficiency and profitability of dairy herds.

REFERENCES

8 Booth, J. M. 1980. Milk progesterone pregnancy...


