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The use of a specific sensitive ELISA test that determines progesterone levels in milk to confirm the correct time of insemination in dairy cattle

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GENERAL INTRODUCTION

Reproductive efficiency is important for the dairy farmer. The lactation curve for the dairy cow reveals higher production levels during the first half of the lactation. The peak and persistency of the production level depends on the age and nutritional and health management of the cow. Prolonged calving intervals allow the cow to produce at a lower level with a loss of lifetime production for the cow and income for the dairy producer (Britt, 1985). This is especially true as herd size and production increases (Spalding et al., 1975). To assure that calving intervals remain at the desired level for a particular dairy farm, the producer must manage the animal to minimize health problems, insure adequate nutrition for the stage of lactation, and minimize stress through proper housing and husbandry practices. This encourages the return to a functional reproductive state early in the postpartum period so breeding and conception can occur in a timely manner (Weaver and Goodger, 1987). Dairy producers need to maintain calving intervals of between 12 and 13 months to assure maximum lifetime production from their cows.

As production increases lactational stress on the cow makes it difficult to maintain reproductive efficiency within a herd. With larger herds the time consuming tasks such as estrous detection often are slighted.

Although there are many reasons for decreased reproductive efficiency, such as postpartum disease and cystic ovaries, the main reason still remains inadequate estrous detection. This can include poor expression of estrus by the cow or failure by the dairy producer to observe estrous activity when it occurs. Estrous detection requires a dedicated effort by all persons involved to assure cows are presented for breeding at the most opportune time for conception.

One of the many heat detection aids available to the farmer is the enzymeimmunoassay (EIA) for progesterone in milk. Progesterone levels are helpful in early pregnancy detection, confirmation of estrus, selecting animals for hormone therapy and many other uses in the reproductive management of the dairy cow. Since progesterone is low at the time of estrus, a test with adequate sensitivity should help to confirm that estrus is near. Multiple samples over several days may be necessary to exactly pinpoint estrus. Tests have been developed which can be quickly and cost effectively performed on the farm.

This study was conducted to evaluate one of the commercial tests which appeared to be sensitive enough to more accurately pinpoint estrus and to determine its usefulness to the dairy producer in making the decision of when to breed the cow.

Explanation of Thesis Format

This thesis is in the alternate format and will consist of a review of the literature, paper in a publishable form, summary and discussion, list of the literature cited and the acknowledgements. References listed in the literature cited are those used in the introduction, literature review and the summary and discussion. Selected references used in the paper have been listed in its bibliography. Figures and tables within the literature review and the paper will be contained within the text in close proximity to where they will be discussed. The data presented in the paper was accumulated from original work performed by the candidate under the guidance of the co-author and the candidate had sole responsibility for the preparation of the manuscript.

REVIEW OF LITERATURE

Reproductive Management

Reproduction plays a key role in the profitability of a dairy herd (Rounsaville et al., 1979; Britt, 1985; Weaver and Goodger, 1987). Dairy farmers are realizing that poor reproductive performance directly increases their expenses and decreases their income. Increased feed costs, cows lost to untimely culling, breeding expenses due to semen costs, heat detection and insemination time, and often a rise in the incidence of peri-parturient diseases all increase expenditures for the farmer. Income is lost due to decreased lifetime milk production, fewer calves to sell or use within the herd and slower genetic progress. The effects of reproductive performance on the economic efficiency of a dairy farm are often not felt immediately but are delayed (Britt, 1985).

Dairy producers are reporting that management of reproduction is one of their most difficult problems (Rounsaville et al., 1979). Many list low herd fertility as their number one problem, although they report no known disease entities within their herd (Spalding et al., 1975). Morrow (1980) states that a calving interval of 365 days (12 months) is optimal; however, Weaver and Goodger (1987) report current Dairy Herd Improvement Association (DHIA) records from 15 states (740,000 cows in 3500 herds) indicate that average

calving intervals exceed 13.3 month. They also indicate that calving intervals in Canada and Australia average 13.2 months. Herds in Iowa on DHIA have an average calving interval of 396 days (13 months). The primary reasons for long calving intervals are delays to first service often due to failure to detect heat, low fertility at breeding, and the late detection of open cows after insemination (Spalding et al., 1975; Morrow, 1980). Other changes in the dairy industry that have affected reproductive performance of dairy herds include increased herd size, more cows per person, enhanced genetic ability for milk production, nutritional demands of cows, and new housing and feeding systems (Spalding et al., 1975)

Heat detection rates vary considerably depending on the dairy herd. Weaver and Goodger (1987) report a range of 30% to 85% between herds. Britt (1985) reports that 10% to 30% of cows are not in estrus when inseminated. Reimers et al. (1985) reports the proportion of cows not in or near estrus when inseminated varies from 0 to 60% among herds. Heat detection depends on the farmer's willingness to observe cows for signs of estrus (intensity) and how good they are at determining if a cow is in estrus or not (efficiency). Heat detection rates can be maintained between 50% and 70% with routine observation for estrus twice daily (Britt, 1985).

The Estrous Cycle

In a normal bovine female of breeding size and age, estrus, which is the period when the female will accept

service by a male, occurs on the average every 21-22 days (Hafez, 1987). Norris (1980) states this is the time when fertilization will most likely lead to pregnancy. If pregnancy does occur the cow becomes anestrus. Occasionally the cow may express estrus when pregnant.

Late in the estrous cycle there is a period of declining blood progesterone level due to the demise of the corpus luteum (CL) while folliculogenesis is preparing a new follicle(s) for maturation and subsequent ovulation. Hafez (1987) describes this as the follicular phase which lasts from 3 to 6 days in cows. Progesterone levels reach their nadir during estrus which lasts 18 to 19 hours. Ovulation normally occurs 10 to 11 hours after the female ceases to be receptive to the male (Hafez, 1987). With ovulation, luteinization of the follicular cavity occurs and the corpus hemorrhagicum (CH) grows quickly both in size and secretory ability. Hafez (1987) defines this period as the luteal phase which lasts 16 to 17 days in cattle. It takes approximately seven days post-ovulation for the CH to become a mature CL; during the first 5 to 7 days of the luteal phase the CH is refractory to endogenous or exogenous prostaglandin. Once mature it will actively secrete progesterone until a luteolytic factor kills it at the end of the luteal phase or there is maternal recognition of pregnancy and the CL is spared so it may remain active for the duration of the pregnancy. This is the period when the highest concentration of progesterone occurs in the

blood (Hafez, 1987). If pregnancy occurs in the cow the CL remains active and secretes progesterone for the duration of the gestation period, declining only when parturition is imminent. Progesterone levels remain low in the prepubertal or acyclic animal.

Progesterone

Progesterone is a steroid hormone secreted in the bovine primarily by the CL but also to some degree by the placenta and adrenal cortex (Hafez, 1987). Niswender and Nett (1988) and Norris (1980) describe several biological effects of progesterone upon its target tissues within the reproductive system. The mucosal lining of the uterus, if it has been exposed to estradiol, will form receptors for progesterone. Progesterone along with estrogen regulates contractions of the oviduct to influence the rate of transport of the ovum into the uterus. This is timed such that the embryo arrives after the uterus has been prepared for its arrival and it will be assured nutritional support. Progesterone contributes to secretory activity by the oviductal epithelium to provide nourishment for the developing conceptus.

Preparation of the uterus for pregnancy is a function of progesterone. Under the influence of estrogen the uterine mucosal epithelium proliferates as does the underlying stroma. Endometrial glands lengthen but remain straight as they extend into the stroma. Progesterone stops the cell proliferation, causing glands to coil and increasing the vascularity within

the stroma. These changes prepare the cells to provide nourishment for the conceptus until attachment and placentation occurs. Progesterone also blocks organized contractions by the myometrium.

Under the influence of progesterone the cervical mucus becomes highly viscus. This effectively blocks the passage of materials into or out of the uterus. Vaginal epithelium thins due to progesterone influence.

Progesterone in conjunction with other hormones such as estrogen and anterior pituitary hormones causes the development of the lobuloalveolar tissue of the mammary gland. This effect appears to occur only when there is prolonged secretion of progesterone such as in pregnancy and is minimal during a normal estrous cycle.

Progesterone inhibits gonadotropin release (Norris, 1980) due to a direct effect on the gonadotropin-releasing hormone pulse indicator in the hypothalamus (Niswender and Nett, 1988). This causes the pulse of luteinizing hormone to become more infrequent and of a higher amplitude than during the follicular phase of the cycle. There is little effect on follicle-stimulating hormone activity.

Milk Progesterone

The corpus luteum, which is a transient endocrine gland located on the ovary, produces progesterone but since it has limited capacity to store it (Niswender and Nett, 1988), progesterone is released rapidly into the circulation in

transit to target organs. Although progesterone was first located in a lipoidal extract of luteal tissue in 1929 and purified and crystallized in 1934 (Niswender and Nett, 1988), its presence in bovine fluids and tissues was identified much later. Pope and Swinburne (1980) reported that progesterone was identified by various researchers in the following bovine tissues and fluids: adrenal gland (1938, 1958); adrenal venous blood (1957); corpus luteum tissue (1953, 1958); non-luteal ovarian tissue (1958); ovarian follicular fluid (1953, 1962); systemic blood (1958, 1967, 1969) and body fat (1963). They state that the first identification of progesterone in bovine milk was by McCracken in 1963 and this was later confirmed by Gupta in 1967. They also felt that while there is no doubt that there is progesterone in milk, there are possibly other closely related steroid compounds present which, although present in smaller quantities, may also be detected by current assay systems and may not be distinguishable from progesterone. This has prompted researchers to attempt to improve the specificity of their progesterone assays.

Bremel and Gangwer (1978, p. 1103) state, "Estrogens and progesterone are in bovine milk in concentrations generally exceeding those in blood. Although milk is not a major excretory route of the hormones, their concentrations reflect concentrations of hormones in blood during the estrous cycle and pregnancy." They propose that nonelectrolytes such as progesterone in the blood are permeable to cell membranes and

should be in the milk in concentrations directly related to that in the blood. Concentrations may actually be higher due to a partition coefficient between the aqueous and lipid phases of the milk. This appears to be true for progesterone. They also felt that the binding proteins may be secreted from the cell leaving the progesterone behind. Bremel and Gangwer (1978) studied cortisol, a steroid hormone, and how it entered the milk compartment after adrenocorticotropin injection in cows to investigate this theory. Regression equations for the transport of cortisol from blood to milk were nearly identical. They took this to mean that the mammary gland integrates the circulating levels of cortisol into milk to reflect the levels in the blood and applied this transfer mechanism to other steroid materials as well. Pope and Swineburne (1980, p. 428) felt that progesterone was present in milk because of a "physiological steady-state equilibria existing between [its] concentration in blood, extra-cellular fluid, mammary epithelial cells and secreted milk." Although the mechanism is not clearly defined it is apparent that progesterone and other hormones readily enter into the milk compartment in detectable amounts. Narendran et al. (1979) felt the high concentrations of progesterone in milk were at least partly due to an active uptake and metabolic role by the functioning mammary gland.

The presence of progesterone receptors in the cytosol from nonlactating, pregnant cows and from lactating,

nonpregnant cows has been reported by Capuco et al. (1982). The nonlactating, pregnant cows had 4 times as many progestin binding sites as did the lactating, nonpregnant cows. They felt that the specificity of their binding sites was definitely for progestin and not for glucocorticoid or corticosteroid binding globulin. Although they reported work stating that progesterone inhibits lactogenesis at the level of the mammary gland, they offered a comment that due to the relative lack of binding sites in lactating tissue there would be little effect of progesterone on inhibiting an established lactation.

Many researchers have studied the relationship between serum or plasma progesterone and milk progesterone to determine if milk concentrations accurately depict the concentrations in serum or plasma. In 1971, Laing and Heap determined the concentration of progesterone in milk and compared these to previous reports of plasma concentrations at different stages of the estrous cycle, anestrus and pregnancy. Known anestrous cows had concentrations of 2.25 $\pm$ 0.51 ng/ml (mean $\pm$ S.E.) while mid-luteal phase cows had mean concentrations of 3.82 $\pm$ 0.76 ng/ml (range=1.4 to 6.05 ng/ml) and pregnant cows had levels of 18.55 $\pm$ 2.20 ng/ml. The levels for non-pregnant and late pregnant cows were similar to plasma concentrations reported by other researchers, but early pregnancy samples in this trial were much higher.

Milk and plasma concentrations for progesterone were monitored by radioimmunoassay during the estrous cycle of four cows by Dobson et al. (1975). They report the mean maximum progesterone level for plasma was 7.5 ± 2.0 ng/ml and 14.7 ± 1.9 ng/ml for milk. Mean low values were 0.3 ± 0.07 ng/ml for plasma and 3.0 ± 0.7 ng/ml for milk. While absolute values were different, there was a close temporal pattern between the two types of samples and the regression coefficient was $r=0.88$.

Hoffman et al. (1976) did a similar study and found that the plasma and milk progesterone concentrations parallel each other but as in the other trials the milk levels were higher than the plasma levels. Lamming and Bulman (1976) found very close parallelism between milk and plasma progesterone values. This prompted Nakao et al. (1983, p. 109) to state, "Milk progesterone level, therefore, is a clear indicator of luteal function."

Although milk progesterone levels seem to be similar to blood levels of the hormone during the stages of the estrous cycle, some exceptions have been reported. In an investigation of 6 herds involving 4 trials, Bulman and Lamming (1979) discovered one herd which had mean progesterone levels of 5.4 ± 1.05 ng/ml on the day of insemination. The 5 herds in the other 3 trials had values of 1.1 ± 0.96 ng/ml, 1.2 ± 0.34 ng/ml, and 1.1 ± 0.13 ng/ml. Despite the

progesterone levels being high the herd had a conception rate of 83% confirmed by progesterone testing and calving data.

In another trial, Dobson et al. (1975) had 1 cow out of 4 sampled have a progesterone level of 7.2 ng/ml on the day of estrus. This was not reflected in the corresponding plasma sample or in milk sampled from the day before or the day after estrus.

Foote et al. (1980) published data in which 13 of 47 cows in their trial had high progesterone levels at the time of breeding. Conception rates for the cows with high progesterone was 8% versus 62% for the cows with low progesterone. They felt the reason for the high samples was improper estrous detection.

Transient peaks of progesterone were found in a number of cows at estrus by Foulkes et al. (1982). They contributed this to progesterone-like materials being detected by the progesterone assay. Enzymeimmunoassays may have more of a problem with this than radioimmunoassays according to these workers.

Up to 5 ng/ml of progesterone can be contained in the milk of estrous cows according to Marcus and Hackett (1986). In a trial involving 960 cows, 18 (1.9%) had milk progesterone values above 8 ng/ml and 14 (1.5%) had values between 3 and 8 ng/ml on the day of estrus according to Pennington et al. (1985). Five of these cows became pregnant at this time of which 3 were in the >8 ng/ml grouping.

In contrast to high progesterone levels at estrus, Jackson et al. (1979) reported an 18% incidence of prolonged low milk progesterone (<2 ng/ml for 8+ days) observed with natural estrus. These cows had a 64% conception rate indicating there was no lowered fertility.

Most of the progesterone in milk is located in the fat portion (Foote, 1979; Ax, 1980). The fat content of milk varies between the fore-milk, composite milk and post-milk strippings, with the lowest fat content in the fore-milk and the highest fat in post-milk. The progesterone in milk varies in direct correlation with the fat content (Pope et al., 1976). The highest progesterone levels are in the post-milk strippings (Foote, 1979; Foote et al., 1979; Pope et al., 1976). While first milk had significantly lower concentrations of progesterone, Pennington et al. (1981) found no difference between composite milk and last milk. Hoffman et al. (1976) compared the levels of progesterone in the fat from different stages of milking (initial, middle, last and whole milking). Although the samples varied in fat content there was no difference in progesterone content when expressed per unit of fat. Fat content varies between cows which significantly affects the progesterone concentration in milk (Pennington et al., 1981). When breed differences in milk progesterone concentration were investigated only the Jersey breed had significantly higher levels in their milk (Pennington et al., 1981).

There is no difference in progesterone levels in milk whether expressing them as progesterone per unit of milk fat or progesterone per ml of milk (Lamming and Bulman, 1976). However to standardize and improve interpretation of test results, various researchers have used different fractions of milk in their testing protocol. Investigators using milk fat for progesterone determination include Pennington et al. (1976), Caudle et al. (1980), Gunzler et al. (1979), and Claus et al. (1983). Caudle et al. (1980) found milk fat progesterone levels to be 12 to 27 times greater than those in milk or plasma. The Gunzler and Claus groups provided milk progesterone assays for farmers through the Bavarian Animal Health Services. They felt milk fat analysis had the advantages of concentrations being independent of sampling techniques, and of being highly repeatable and sensitive making interpretation easier, thus minimizing incorrect interpretations.

Other investigators have used the skim milk portion of the sample for much the same reason (Nakao et al., 1982; Nakao et al., 1983; McCaughey and Gordon, 1979), although the progesterone levels in defatted milk were found to be much lower than in whole milk (McCaughey and Gordon, 1979). Other groups have found whole milk to be suitable (Lamming and Bulman, 1976; Pope et al., 1976; Foote et al., 1979). MacFarlane et al. (1977) used the fore-milk from suckling

beef cows collected one-half hour before the calf was allowed to nurse to determine pregnancy by milk progesterone levels. Their findings were similar to others using whole milk or fat samples.

Milk progesterone was found to be higher in the evening milk than in the morning milk by Thibier et al. (1976). There were only random differences between quarters of the mammary gland concerning progesterone levels in the milk (Foote et al., 1979; Ax, 1980). McCaughey and Gordon (1979) in their trial utilizing defatted milk for progesterone assay found no significant difference between quarters.

Laitinen (1986) studied the effects of mastitis and milk composition on milk progesterone. He concluded that milk progesterone, fat and total protein levels were not affected by mastitis. In contrasting work, Wimpy et al. (1986) found that milk samples with somatic cell counts above 323,000 gave high progesterone readings in nonpregnant cows when sampled 21 days after insemination This caused them to be falsely classified as pregnant and lowered the accuracy of pregnancy detection using 21 day milk samples to 51%. This compared to 78% and above in cows with somatic cell counts below 318,000. Both groups used post-milk strippings, however Laitinen used radioimmunoassay while Wimpy and associates used enzymeimmunoassay.

It is often impossible to assay milk samples for progesterone immediately after the sample is taken. Samples

will spoil within a few hours or days unless properly cared for. Spoiled samples will separate into a liquid portion and a precipitate. The progesterone will be associated with the precipitate (Pope and Swinburne, 1980). Samples can be used if the curdled milk is thoroughly homogenized (Foote et al., 1979). Several methods have been proposed for preservation and storage of samples.

Samples can be frozen at -10^0C but Heap et al. (1976) noted that deterioration of some samples still took place. Dobson et al. (1975) found that storing samples at -15^0C had its disadvantages. Upon thawing there was precipitation of solids from the milk and assay of the supernatant gave erratic results. Freezing defatted milk samples for 3-6 months without preservative results in separation into a clear liquid and flocculent precipitate after thawing according to Pope and Swinburne (1980). They overcame this by adding 7.5% 0.5M-EDTA at pH 7.0 to the sample after thawing with no effect on radioimmunoassay.

Potassium dichromate and mercuric chloride were commonly used as milk preservatives by researchers (Foote et al., 1980; Jackson et al., 1979; Davies et al., 1987; Dobson et al., 1975; Heap et al., 1976). They felt that preserved samples could be stored at room temperature for several days and at 5^0C for several months with no ill effect on the assay. This preservative also appears to improve the storage of samples at -10^0C to -20^0C.

Sodium azide was used by Epelu-Opio and Madej (1988) to preserve their samples at 4^0C. Precipitates can form with this preservative which may be detrimental to assay results (Pope and Swinburne, 1980). Other alternative preservatives include formaldehyde and antibiotics (Pope and Swinburne, 1980).

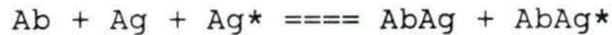
Pennington et al. (1981) studied the effects of storage on milk samples. They divided milk samples into two groups, one with no preservative and one with potassium dichromate as the preservative. Representative samples were assayed at one hour for progesterone. Other samples were assayed at five and ten days for progesterone. They were stored as follows: -10^0C, -10^0C for one day then at room temperature (22^0C), 4^0C, room temperature, and 37^0C. Progesterone concentration was not affected by days of storage, method of storage or addition of a preservative.

Progesterone Assays

Various methods have been used to measure milk progesterone including mass spectrometry, gas chromatography, competitive binding assays, radioimmunoassay (Mather et al., 1978) and more recently enzymeimmunoassay (Arnstadt and Cleere, 1981; Sauer et al., 1982; Nakao et al., 1982). Immunoassays have become the most common method with over 70 million assays performed in 1983 and numbers are expected to rise until upwards of 1 billion are performed each year. They have the advantage of being, in theory, applicable to any antigenic compound since only specific antibody is needed;

they are highly sensitive; and they require little or no pretreatment of the sample (Blake and Gould, 1984).

The principle of immunoassay is relatively simple and is shown by the following equation:



$$Ab + Ag + Ag^* ==== AbAg + AbAg^*$$

where Ab is the specific antibody, Ag is free antigen in the sample, and Ag* is the labeled antigen. After incubation the free and antibody bound antigen are separated and the quantity of the labeled antigen is measured. The higher the concentration of unlabeled (free) antigen in the sample, the less binding of labeled antigen to antibody will occur. This is known as competitive inhibition. The amount of labeled antigen is measured and standardized curves, that have been previously determined, are used to quantify amounts of antigen in the sample (Blake and Gould, 1984).

Radioimmunoassay (RIA) utilizes antigen bound to a radioactive label which can be detected by a counting chamber. This requires the handling of radioactive materials and sensitive radioactive detection devices. Other disadvantages include the production, storage and disposal of radioactive materials; the expense of setting up and maintaining a lab; extraction steps in the procedure are difficult to automate; and the need for a centralized laboratory which makes on-site determinations impossible, and requires licenses and skilled technicians (Blake and Gould, 1984; Nebel et al., 1987). Several central labs using RIA technology have been

established to measure milk progesterone (Booth and Holdsworth, 1976; Gunzler et al., 1979; Cidoncha and Garcia, 1982). RIA is also very useful for monitoring luteal function in species other than the cow (Srikandakumar et al., 1986).

The need for rapid on site testing of samples has led to the development of alternative testing procedures of which enzymeimmunoassays (EIA) have become the most popular. Their extreme versatility has led to a variety of products that detect hormones, infectious agents, antibodies, antibiotics and many others with ease and simplicity, These tests can be performed in clinics, hospitals, small labs and even on the farm.

EIA allows for the detection of specific products within samples without the use of radioactive labels. In this technology the test antigen or antibody is labeled with a substance that will react with an enzyme to produce a specific reaction. The results of the reaction can then be detected by a change in the substrate media, examples of which are color changes or agglutination. Results can be qualified or quantified. Comparison of EIA to RIA is given in Table 1.

The principles and variations of EIA are given in Figures 1-4. Figure 1 represents classical EIA which is similar to RIA. Competitive binding for a limited amount of antibody is allowed to occur or a variation is used in which free antigen is allowed to interact with antibody for a specified period of time after which labeled antigen is added. Labeled antigen

antibody complex is separated from free antigen antibody complex and allowed to react with the enzyme (Blake and Gould, 1984).

Table 1. Comparison of enzyme with radiolabels in immunoassay (Blake and Gould, 1984)

(a) Advantages of enzyme labels
- (i) No radiation hazards occur during labeling or disposal of waste.
- (ii) Enzyme-labeled products can have a long shelf life, e.g. 1 year or more.
- (iii) Equipment for enzyme assay can be inexpensive and generally available.
- (iv) Homogenous assays can be completed in a few minutes and are readily automated.
- (v) Heterogenous assays are ideal for visual qualitative tests.
- (vi) Multiple simultaneous assays are possible.

(b) Disadvantages of enzyme labels
- (i) Plasma constituents may affect enzyme activity.
- (ii) Assay of enzyme activity can be more complex than measurement of some types of radioisotopes.
- (iii) Less control of enzyme labeling reactions.
- (iv) At present homogenous EIAS have limited sensitivity.

Figure 2 illustrates the competitive enzyme-linked immunosorbent assay for antigen. Here the antibody is labeled and allowed to react with free antigen and solid-phase antigen. Free antigen antibody complex is removed and solid-phase antigen bound to labeled antibody is measured. This process can be reversed where the antigen is labeled and the antibody is the solid-phase (Blake and Gould, 1984).

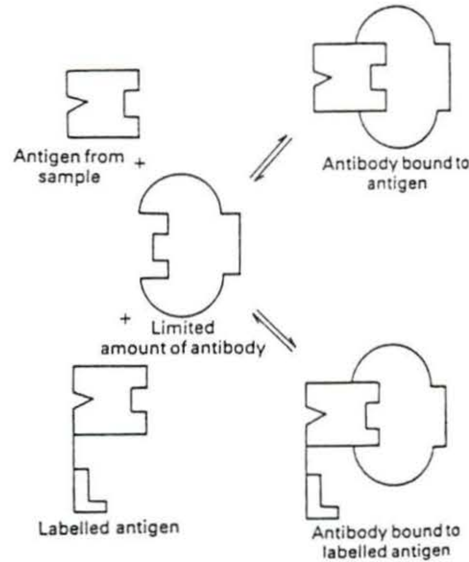


Figure 1. Classical competitive EIA for antigen. All components are mixed with a limited amount of antibody specific for the antigen. Separation of of enzyme activity (Blake and Gould, 1984)

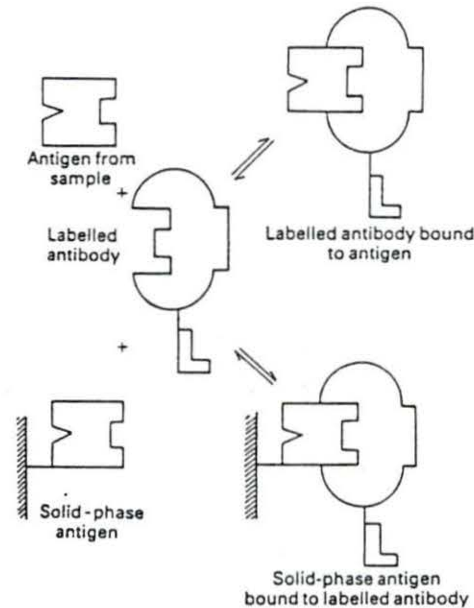


Figure 2. Competitive ELISA for antigen. Enzyme-labelled antibody reacts specifically with antigen in the sample and is then added to excess of solid-phase antigen. After washing, the enzyme label attached to the solid phase is measured (Blake and Gould, 1984)

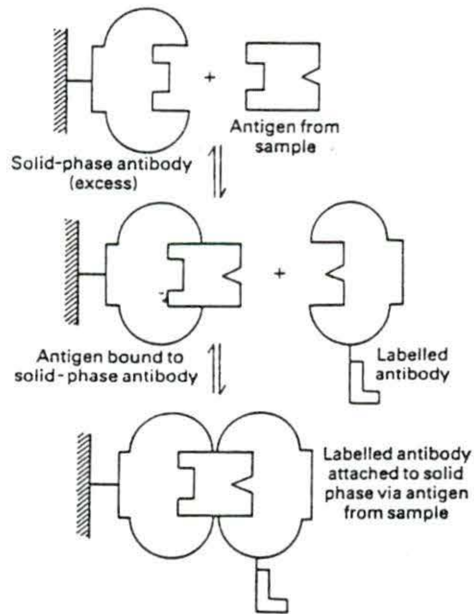


Figure 3. Sandwich assay for antigen. Antigen in the sample is mixed with excess of solid-phase antibody. After washing of the solid phase, enzyme labelled antibody, which is specific for another site on the antigen, is added. The enzyme label, which remains bound after washing, is measured (Blake and Gould, 1984)

In the sandwich assay for antigen (Figure 3), solid-phase antibody reacts with antigen from the sample. After washing the solid-phase antibody free antigen complex is mixed with labeled antibody which is specific for another site on the antigen. After another wash the remaining bound labeled antibody is measured (Blake and Gould, 1984).

A variation of the sandwich assay is for antibody in the sample (Figure 4). Solid-phase antigen is interacted with antibody in the sample. After washing, labeled antibody produced for another site on the free antibody is added. The

sample is again washed and the labeled complex is measured (Blake and Gould, 1984).

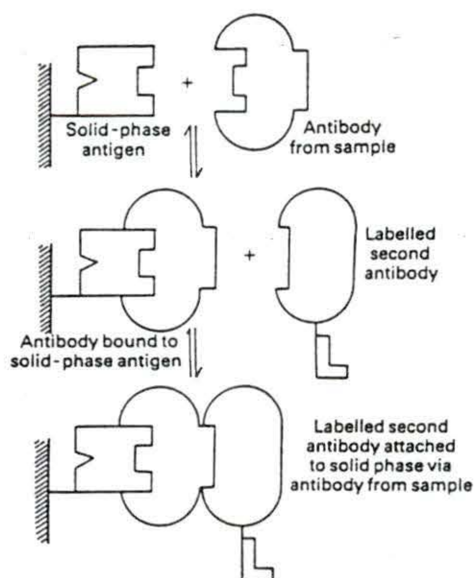


Figure 4. Sandwich assay for antibody. Antibody in the sample is mixed with excess of solid-phase antigen. After washing of the solid phase, enzyme-labelled second antibody is added. Bound enzyme quantity is measured after washing (Blake and Gould, 1984)

Many groups have reported applying the EIA technology to developing tests for milk progesterone (Arnstadt and Cleere, 1981; Chang and Estergreen, 1983; Munro and Stabenfeldt, 1984; Cleere et al., 1985). Others have suggested changes to EIA that improve the accuracy when assaying milk. These include the use of double antibody (Prakash et al., 1986), monoclonal antibody (Ellis et al., 1986) and enzyme-amplified immunoassay (Worsfold et al., 1987).

Several workers have checked the validity of EIA for the determination of milk progesterone levels by checking it

against RIA. They have found EIA to be simple, accurate and reliable for this procedure (Sauer et al., 1981; Sauer et al., 1982; Nakao et al., 1983; Van De Wiel and Koops, 1986; Sauer et al., 1986).

The commercial EIA tests available for milk progesterone determination have been evaluated (Davies et al., 1987; Elmore, 1989), reviewed (Elmore, 1986; Nebel, 1988), and compared (Nebel et al., 1989). The tests have been found to be simple and convenient to use in the lab or on the farm. If one understands the shortfalls of these tests, the information provided by them is useful in the management of bovine reproduction. Davies and Fletcher (1987) also evaluated a commercial kit for progesterone in bovine blood and found it to be accurate. Other researchers suggest uses in other species (Booth, 1980; Cleere et al., 1985; Bretzlaff et al., 1989).

Uses for Milk Progesterone

Much work has been cited describing the many uses for determining progesterone levels in milk. In the bovine female these uses center around reproductive management although some are valuable for the researcher.

Diagnosis of pregnancy

A major thrust for the use of milk progesterone has been for the early diagnosis of pregnancy. While the most common method for determining pregnancy in the bovine is by rectal palpation after approximately 35 days postbreeding, milk progesterone can be

used at the next expected heat at 21 days postbreeding. It has been shown that progesterone levels in the pregnant cow remain elevated at the next expected heat (Ax, 1980). A milk sample taken at 21 to 24 days after breeding that has a high diestrous progesterone concentration suggests that the cow is pregnant (Morrow, 1980). A summary of the accuracy for pregnancy diagnosis is presented in
Table 2.

Table 2. Accuracy of pregnancy diagnosis from milk progesterone

Accuracy for Diagnosing Pregnancy	Accuracy for Diagnosing Non-pregnancy	Reference
80%	95-100%	Pope et al. (1976)
80%	98%	Foote et al. (1979)
83.5%	95.3%	Pennington et al. (1985)
77.5-85.8%	85.7-100%	Heap et al. (1976)
94.6%	100%	Macfarlane et al. (1977)
81%	91%	Dobson and Fitzpatrick (1976)
96%	89.6%	Marcus and Hackett (1986)
97%	98%	Laing et al. (1980)
83.5%	97%	Wishart et al. (1975)
60%	100%	Nakao et al. (1982)
80%	98%	Foote et al. (1980)
77%	97%	Hoffman et al. (1976)
84.5%	97%	Booth (1980)
85.1%	98.8%	Cidoncha and Garcia (1982)

The groups that had the highest accuracy for determining pregnancy utilized multiple samples, namely day 0 and day 21 (Marcus and Hackett, 1986), 3 samples 8 days apart (Laing et al., 1980), and day 21 and day 24 (Wishart et al., 1975).

Nakao et al. (1982) attributed a 27.9% incidence of embryonic mortality for the low accuracy of diagnosing pregnancy in their study.

The reasons listed by Clark and Bierschwal (1986) for the low accuracy of diagnosing pregnancy by progesterone level include:

1. Insemination during the luteal phase
2. Long or short estrous cycles
3. Prolonged luteal lifespan (uterine pathology)
4. Embryonic death (after day 16 or 17)
5. Ovarian cysts
6. Management errors (mistiming of sample collection, mistaken identification of cow and/or sample)
7. Laboratory errors

Lynch and Abbott (1988) suggest that a progesterone test performed on day 19 after insemination is very accurate at detecting the non-pregnant animal.

Monitor postpartum cycling   Anestrus in the postpartum period can be due to acyclicity, ovarian cysts, uterine involution disturbances (Heinonen et al., 1988), or severe weight loss due to high production or nutritional inadequacies. Often it is difficult to determine if a cow has commenced cycling even with rectal examinations. Progesterone profiling where samples are taken twice a week (Ax, 1980; Bulman and Lamming, 1978) proved useful for confirming cyclicity. Morrow (1980) reported that progesterone profiles on 1292 cows revealed a 25% incidence of subfertility in the form of non-detected estrus, inactive ovaries, and embryonic death. Ball (1982), Mather et al. (1978) and Gunzler et al.

(1979) used progesterone profiling to study the onset of ovarian activity in the postpartum cow. Samples taken at a 10 day interval at 50 and 60 days postpartum proved to be accurate in detecting cyclicity for Heinonen et al. (1988).

Confirming the time to breed   Estrous detection is a difficult and frustrating task for the dairy producer. Any tool that improved the accuracy of selecting cows for breeding and was cost effective would be welcomed. By using milk progesterone Ball (1982) and Ball and Jackson (1978) noted that some cows had regular ovarian cycles but failed to show estrus. Others have shown that many cows selected for breeding are actually in the luteal phase of the cycle and not suitable for insemination (McCaughey and Cooper, 1980; Claus et al., 1983; Gunzler et al., 1979; Clark and Bierschwal, 1986). Eddy and Clark (1987) found an ELISA progesterone test in cows useful for predicting estrus, helpful in improving estrus detection and capable of detecting the return to estrus after service.

Progesterone assays of milk samples are usually not sensitive enough to detect estrus or time to breed using only one test result. Varner (1986) proposed the daily collection and analyzation of samples around the time of expected heat and breeding once at 48 hours post progesterone decline and again at 72 hours post progesterone decline. Eddy (1983) proposed a breeding scheme that utilized prostaglandin injections, milk progesterone assay and rectal exams to breed

difficult to conceive cows. The following scheme was proposed by Elmore (1987) for breeding cows without estrous detection.

Monday: Milk Progesterone Performed
A. High: prostaglandin and breed at 72 and 96 hours
  1. 21 days later: milk progesterone performed
    a. high: palpate at 35 days for pregnancy
    b. low: milk progesterone sample next Monday
B. Low: milk progesterone performed next Monday

Milk progesterone to screen for subfertility Lamming (1980) found that 25% of the cows he tested using milk progesterone had subfertility in the form of delayed start to ovarian cycles, cessation of cycles, prolonged luteal activity, silent estrus and embryo mortality. Others have suggested its use as well for detecting and for treating subfertility (Dobson and Fitzpatrick, 1976; Lamming and Bulman, 1976; Ax, 1980). This requires at least twice a week sampling and progesterone assay. By doing this the pattern of progesterone secretion can be determined for an individual cow and an assessment of ovarian activity made (Varner, 1986). Timely treatments, changes in diet and preventative measures for herd problems (e.g. postpartum diseases) can be implemented. In spite of all the reasons for subfertility, Ball (1982), in an evaluation of over 1400 progesterone profiles, found the biggest single cause of delay in insemination to be failure to detect estrus. He found milk progesterone useful in convincing dairymen that failure to detect estrus is a problem and in using it as an educational

aid to improve estrus detection. Laing (1976) used milk progesterone testing to help control infertility.

Milk progesterone to evaluate anestrous cows Anestrus is composed of two components which are often confused. Cows may be acyclic meaning they have no ovarian activity leading to normal regular estrous cycles and they can be anestrus which means they fail to express estrus. Components of anestrus include silent estrus, acyclia, pregnancy, pre-puberty, ovarian cysts and endometritis (Heinonen et al., 1988).

Claus et al. (1983) studied anestrus and found 15.8% of the cows had ovarian cysts and 10% had corpus luteum cysts (with or without endometritis). They found acyclia was longer in the spring compared to other seasons and in stanchion barns compared to loose housing. Varner (1986) proposed that paired milk samples, taken at 27 and 35 days postpartum, be used in conjunction with rectal palpation to determine the rates for cystic ovaries and anestrus within a herd. In another study anestrous cows were detected using milk progesterone sampling and appropriate therapy was applied and assessed (Lamming and Bulman, 1976).

Milk progesterone and repeat breeders Repeat breeders offer a unique challenge to the farmer and veterinary practitioner. Often no physical abnormalities can be found and examination via rectal palpation discovers luteal structures on the ovaries. Milk progesterone can be utilized

in these animals to accurately time hormonal treatments since milk progesterone trials have shown many to have abnormal patterns of progesterone secretion (Lamming and Bulman, 1976; Bulman and Lamming, 1978).

Milk progesterone and embryonic mortality Embryonic mortality occurring after 21 days of gestation is a major problem in dairy herds (Morrow, 1980). Macfarlane et al. (1977) considered embryonic mortality as a factor lowering the accuracy of pregnancy diagnosis by milk progesterone. Other workers have reported the following incidence of embryo mortality in their trials: 12% (Bulman and Lamming, 1979); 16.9% (Claus et al., 1983); 27.9% (Nakao et al., 1982); 7.2% (Foote et al., 1979).

Milk progesterone and ovarian cysts Kesler and Garverick (1982) reviewed ovarian cysts and found them to be a serious cause of reproductive failure. Classically ovarian cysts are diagnosed by rectal palpation but often it is difficult to differentiate between follicular cysts and partially luteinized cysts. Sprecher et al. (1988) found rectal palpation unreliable for a definitive diagnosis of ovarian cysts. Nakao et al. (1983) found rectal palpation unreliable in determining if treatment was successful. Both groups along with Sprecher and Nebel (1988) encourage the use of milk progesterone assay to define the type of cyst and to aid in the selection of the proper therapeutic hormone. Leslie and Bosu (1983) on the other hand found rectal

palpation accurate in defining luteal cysts but less accurate in detecting follicular cysts. In the evaluation of two hormones for the treatment of cystic ovaries in dairy cows, Dinsmore et al. (1987) used milk progesterone assay to determine response to therapy.

Milk progesterone to evaluate to hormone therapy The practitioner often must wait for an observation by the dairy farm personnel before they know whether an animal has responded to specific hormonal therapy. Milk progesterone offers the advantage of determining changes in the hormonal profile corresponding to the hormone used. Examples include a decrease in progesterone following prostaglandin use, an increase in progesterone after treatment for cystic ovaries with GNRH or LH, and an increase after treating anestrus with GNRH or progesterone products. The following researchers have found milk progesterone assay useful to determine response to these drugs:

1. Prostaglandin: Eddy (1983); Jackson et al. (1979); Hoffman et al. (1976); Gunzler et al. (1979)
2. HCG: Hoffman et al. (1976); Gunzler et al. (1979)
3. GNRH: Hoffman et al. (1976); Gunzler et al. (1979); Lamming (1980); Foote et al. (1979); Nakao et al. (1983); Dinsmore et al. (1987)
4. PRID: Lamming (1980)

Miscellaneous uses for milk or blood progesterone Parker et al. (1988) found they could predict calving time using plasma progesterone. They present a system for deciding a surveillance regimen for valuable animals.

Superovulation of embryo transfer donors has a great degree of variability because of unpredictable ovarian response. Gunzler et al. (1979) and Herrler et al. (1990) have evaluated milk progesterone as a tool to assist in evaluating embryo transfer donors. Monitoring progesterone levels to eliminate undesirable donors or using them to determine proper timing of the superovulation attempt has resulted in donors yielding more corpora lutea resulting in more transferable embryos.

Foote et al. (1979) compared milk progesterone to the use of a vaginal probe designed for the detection of estrus. The probe monitors the changes in electrical conductivity of cervical mucus. Overall both tests were in good agreement although some individuals varied considerably. Both tools proved useful in detecting abnormal ovarian findings such as cystic follicles or persistent corpora lutea.

As already noted Sprecher et al. (1988) and Nakao et al. (1983) found rectal palpation inaccurate in defining the type of ovarian cyst and the response to treatment. On the contrary Van de Wiel et al. (1979) found good agreement when milk progesterone profiles were compared to clinical findings. In a 1988 trial, Heinonen studied the relationship between rectal examinations of the corpus luteum and milk progesterone. He found a single rectal exam during both the normal and the short luteal phases is an unreliable method for

determining the stage of the cycle and the responsiveness to prostaglandin therapy unless other information is available.

Using serum progesterone levels, Ott et al. (1986) found that experienced palpators were correct 50% of the time when progesterone levels are low and 89% of the time when progesterone levels are high. It is apparent that defining the luteal phase of the estrous cycle by rectal examination is more reliable than defining the follicular phase of the cycle.

Economic Assessment

Models for the economic assessment for the use of milk progesterone assays in the early detection of pregnancy have been proposed. Pitcher and Galligan (1990) formulated a decision tree in which milk progesterone assay at day 21 post-breeding was compared to rectal palpation for pregnancy after 35 days post-breeding. They followed cows for 42 days and determined the cost to the producer for testing and days open if the cow had not become pregnant to the initial breeding. They took into account the cost of an individual milk progesterone assay and the accuracy of the test in determining pregnancy. They also discussed the expected cost benefits for using milk progesterone testing in herds where the conception rate of the individual dairy is known. They provided an indifference curve, which utilized the cost of a day a cow is not pregnant and the conception rate and compares these to three different costs for a progesterone test, to assist the

producer in deciding whether milk progesterone testing is cost beneficial or not.

Oltenacu et al. (1990) compared milk progesterone testing at day 19 postservice to uterine palpation for pregnancy on days 35, 50, and 65 postservice. They found a return of $10.50 per cow when milk progesterone testing was used to determine the nonpregnant cow and if these cows were then treated with prostaglandin if not seen in estrus by 10 days after the test was performed. Rectal palpation on day 35 or 50 produced a return of $5.10 or $2.50 per cow respectively when the nonpregnant animal was identified and equipped with a pressure sensitive device for the detection of estrus. Rectal palpation on day 65 postservice produced no cost benefit for the producer.

SECTION I. THE USE OF MILK PROGESTERONE TESTING AS AN AID IN THE DECISION TO BREED THE DAIRY COW

The use of milk progesterone testing as an aid in the decision to breed the dairy cow

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ABSTRACT

Milk samples were collected from six Midwest dairies at the time of insemination and assayed for progesterone using a commercially available ELISA kit. Samples were assigned a progesterone level based on the color of the filtered disk according to a color chart provided by the company (<2.0 ng/ml, 3.25 ng/ml, 5.5 ng/ml, and >8 ng/ml). Cow breeding information and conception data were collected and compared to progesterone level at the time of breeding. There was no significant difference in conception rates between progesterone levels within individual farms and across all farms. There was a significant difference in conception rates between herds. This may have resulted from using postmilking samples which put cows in the high range when they were really low or due to the inaccuracy of detecting high progesterone levels. A high percentage of cows were in the medium to high progesterone level. Reasons for this would include using postmilk or cows being bred when not in heat. Conception data at higher progesterone levels did not support this. Under the experimental conditions of taking milk samples at breeding, this data dids not support the use of this particular milk progesterone test as an aid to the dairy producer in breeding decisions.

INTRODUCTION

The detection of estrus in dairy cattle is a difficult task. Inadequate heat detection lengthens first service interval, days open, and calving interval (Rounsaville et al., 1979; Barr, 1974). Often no male animal is present to identify estrous females for the producer since artificial insemination has become the only method of semen transfer on many dairy farms. This means the producer, their family or employees must be capable of observing estrus so the animal can be presented for breeding at the proper time. Some factors complicating estrous detection include weak or silent estrus, overcrowding, improper identification, failure to recognize signs of estrus, insufficient time allowed for interaction between cows, environment, insufficient time spent observing estrus, nutritional insufficiencies, or postpartum medical problems. For these reasons many cows are either not identified when in estrus or are incorrectly presented for breeding when not in estrus or when already pregnant (Reimers et al., 1985). Many heat detection aids have been proposed and marketed since the producer often fails to see the primary sign of estrus which is standing to be mounted by another animal. Conception rates are much higher when animals are bred to the primary sign of estrus rather than to secondary signs (Reimers et al., 1985).

The determination of the progesterone level of the cow gives a valuable clue as to the stage of the estrous cycle the cow is in (Morrow, 1980). Since it was discovered that progesterone is present in milk as well as in the blood in levels corresponding to the stage of the estrous cycle, this medium has been used for progesterone concentration determination (Foote, 1979).

Many laboratories utilize radioimmunoassay (RIA) to determine progesterone levels but this system requires special laboratory facilities and equipment, licenses, and skilled technicians. The results are often delayed and the disposal of test materials is a problem (Nebel et al., 1987). However, this test is still the standard for research purposes and in some countries the Milk Marketing Boards provide the service to farmers for progesterone level determination (Booth and Holdsworth, 1976; Foote et al., 1980). The advent of the ELISA technology allows for the simple and rapid determination of progesterone levels in milk with a satisfactory degree of accuracy. These tests are cost effective and can be performed quickly on the farm without cumbersome or expensive equipment (Nebel, 1988). The results are often available within minutes.

Since progesterone levels decrease, reach their nadir, and increase in amounts before, during, and after estrus, the sensitivity of the test used determines how accurately estrus itself can be identified. In this study a milk progesterone

test was chosen because it appeared to have a colorimetric system with a low threshold standard of <2.0 ng/ml which might more accurately define estrus. This would assure that cows were being properly identified as being in estrus and inseminated at the correct time. The cost of breeding would be saved in the animals found not to be in heat.

The objective of this study was to assay milk samples for progesterone levels from cows being bred and compare these levels to pregnancy results to see if this test would be useful in helping dairy producers make breeding decisions.

MATERIALS AND METHODS

Six dairy herds in central Iowa were selected for this trial. They ranged in size from 53 to 424 cows. Two were university owned herds with hired managers and the other four were privately owned commercial dairies, two of which had owner/managers and two of which had hired managers. Herd 1 had Guernsey cows; herd 5 had cows representing the common dairy breeds, Holstein, Jersey, Ayrshire, Milking Shorthorn, Guernsey, and Brown Swiss; and the other four herds were Holstein herds. The producers were encouraged to select cows for breeding as they normally would and to breed at their normal time. Five of the herds used observation of estrus for cow selection while one of the herds used a combination of a heat detector animal and observation to identify cows to breed.

At the time of breeding the manager obtained a milk sample, collected from more than one quarter, into a polystyrene tube containing a preservative (potassium dichromate). Although the test directions specified that the milk be sampled from the foremilk or milk jug, this collection time was chosen to be as convenient for the producer as possible. The tube was sealed with an air tight cap and identified as to time of insemination, farm and animal name or number. It was then placed into a refrigerator for storage. Milk samples were collected weekly and returned to the lab.

Milk samples were assayed for the level of progesterone using an ELISA milk progesterone test.[1] The test includes a low progesterone control sample that contains <2ng/ml of progesterone. The control for the high level of progesterone is built into the kit since the filter remains white at progesterone levels >8 ng/ml. A low progesterone control was run for each four milk samples assayed. The test was performed according to instructions supplied by the manufacturer. The level of progesterone was determined by a color change with an intense blue being low progesterone (<2ng/ml) and the test filter remaining white interpreted as high progesterone (>7.25ng/ml). Various shades of blue in between these two colors indicated varying levels of progesterone. The company supplied a color chart that was calibrated to progesterone levels of <2.0, 3.75, 5.5, and >7.25 ng/ml. The samples were read at five minutes according to the instructions and the color on the test's filter was compared to the color chart and the control and the corresponding progesterone level was recorded for each sample.

Herd breeding and conception information were collected. Progesterone levels at the time of breeding were compared to conception results.

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[1]Target, Biometallics, P.O. Box2251, Princeton, NJ 08543

Data was analyzed using the Chi Square test for marginal homogeneity of multiple populations outlined in the Statistical Analysis System.[2]

[2] SAS Institute, Cary, NC.

RESULTS AND DISCUSSION

Table 1 gives the distribution of samples across the farms by progesterone levels. Herd 3 had 66% of samples in the <2.0 ng/ml group and 89% in the two lowest progesterone groups. In contrast the other farms ranged from 30 - 41% in the lowest progesterone level and 52 - 60% in the lowest two categories. Across all farms there were 36% at <2.0 ng/ml, 23% at 3.75 ng/ml, 18% at 5.5 ng/ml, and 23% at >7.25. Samples were expected to be low in progesterone since they were collected at estrus. In this study there was a disproportionate number of samples with medium to high progesterone levels. Possible reasons for this were cows are being selected for breeding when not in estrus or using postmilk instead of foremilk or bucket samples. This would result in increased progesterone levels and thus increased numbers of false positives.

Table 2 shows conception information by farm and progesterone level at breeding. Conception rates at the respective progesterone levels of <2.0 ng/ml, 3.75 ng/ml, 5.5 ng/ml, and >7.25 ng/ml for each farm were Farm 1: 42.9, 20.0, 44.4, and 53.8; Farm 2: 18.2, 00.0, 40.0, and 14.3; Farm 3: 45.2, 54.5, 50.0, and 33.3; Farm 4: 43.2, 39.3, 47.6, and 48.4; Farm 5: 26.7, 31.0, 45.5, and 37.1; Farm 6: 30.0, 33.3, 34.3, and 17.1. There is no significant difference (P>0.10) in conception rates within a farm across progesterone levels.

Table 1. Progesterone readings from milk samples by farm

Farm	Progesterone Level (ng/ml) <2.0	3.75	5.5	>7.25	Total
	n				
1	14	10	9	13	46
2	11	4	5	7	27
3	31	11	2	3	47
4	74	56	42	62	234
5	45	29	22	35	131
6	60	45	35	35	175
Total	235	155	115	155	660

Table 2. Pregnancy percentage by progesterone level and farm

	Progesterone Level (ng/ml) ≤2.0			3.75			5.5			≥7.25		
Farm	O	P	%P	O	P	%P	O	P	%P	O	P	%P
	--n--			--n--			--n--			--n--		
1	8	6	42.9	8	2	20.0	5	4	44.4	6	7	53.8
2	9	2	18.2	4	0	00.0	3	2	40.0	6	1	14.3
3	17	14	45.2	5	6	54.5	1	1	50.0	2	1	33.3
4	42	32	43.2	34	22	39.3	22	20	47.6	32	30	48.4
5	33	12	26.7	20	9	31.0	12	10	45.5	22	13	37.1
6	42	18	30.0	30	15	33.3	23	12	34.3	29	6	17.1

O= Open, P= Pregnant, %P= Percent Pregnant

Since progesterone levels are at their lowest during estrus, it was expected that a much larger percentage of the samples would be in the low range because they were collected at the time of breeding. These conception rates discount a misidentification of estrus as the only source of error. This leaves the test or the technology itself as the reason for the unexpectedly large number of high progesterone levels. The technology has been examined quite extensively and where EIA kits were compared to RIA in determining high levels of progesterone there was an accuracy of 74.8% to 85.6% reported (Nebel et al., 1989). Much of this work was done when progesterone levels were expected to be high such as early pregnancy. Looking at progesterone levels during a narrow window of the estrous cycle such as estrus itself may compound this inaccuracy. Many cows are inseminated after milking and samples taken at this time would have a higher fat content raising the progesterone level, thus increasing false positives for high progesterone.

Figure 1 depicts the average conception rate for each of the four progesterone levels using pooled data from all six farms. Conception rates were 35.7%, 34.8%, 42.6% and 37.4% at progesterone levels <2.0 ng/ml, 3.75 ng/ml, 5.5 ng/ml and >7.25 ng/ml respectively. There was no statistical difference (P>.19) between conception rates of different progesterone levels.

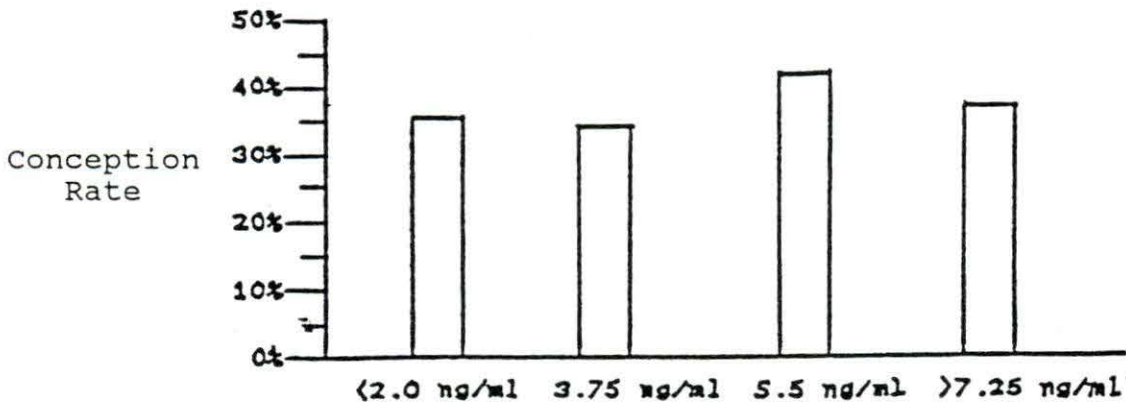

Figure 1. Conception rate by progesterone level.

Conception rates by farm are shown in Table 3 along with herd and sample size. Farms 1, 3 and 4 compared favorably with conception rates of 41.3%, 46.8% and 44.4% respectively. Farm 2 with a conception rate of 18.5% relied almost entirely upon a single individual, the herd manager, to detect cows in estrus. This farm was plagued by consistently low conception rates most likely due to the inability of the manager to detect cows in estrus and to a higher percentage of breedings being performed on cows with presumptive signs of estrus rather than standing estrus. Farms 5 and 6 had additional hired personnel besides the managers who were instructed to observe cows for estrus. Farm 6 utilized a gomer animal equipped with a chin-ball marker as well as observation to detect cows in estrus. Farm 4 was the largest dairy in this trial and had one of the best conception rates. This farm

utilized an incentive program where employees were paid a bonus for each cow they detect in estrus. Farm 4 was a family unit that relied heavily on close observation for the detection of estrus and also believed in giving the cow adequate opportunity to express estrus. There was a significant difference ($P<.05$) between the conception rates of Farm 2 and Farm 3. Farm 4 had a significantly higher conception rate($P<.01$) compared to Farm 6. The overall conception rate for the six farms was 37.1%.

Table 3. Herd size, sample collection size, and conception rate for each herd during the trial

Herd	Herd Size	Number of Samples	Conception Rate
1	65	46	41.3%
2	53	27	18.5%
3	65	47	46.8%
4	424	234	44.4%
5	165	131	33.6%
6	151	175	29.1%

There was a definite difference between the ability of some of these producers to detect estrus and select cows for breeding. Heat detection is a difficult task for the dairy person and errors include failure to observe estrus as well as to misidentify pregnant cows as being in heat (Gunzler et al.,

1979; McCaughey and Cooper, 1980). Milk progesterone has been found useful in determining whether a cow is in heat or not (Eddy and Clark, 1980).

The test used in this trial had a cup which held a membrane that had been treated with progesterone monoclonal antibody. The antibody was attached in a unidirectional manner to orient the active sites outward into the region where the binding reaction takes place.[3] Since the fat portion of the milk contains higher progesterone concentrations (Foote, 1979; Ax, 1980) and since postmilk has higher levels of fat than foremilk or a combined milk sample, the use of the milk taken at the time of insemination may have more closely resembled postmilk due to husbandry practices. Nebel et al. (1989) stated that high fat concentrations in milk blocked the binding of progesterone in the test system used here which lowers the progesterone level. This did not appear to be true for this study.

In this study some of the milk samples stained the filter containing the membrane more than others suggesting some element in the milk was being retained by the filter. It did not appear to be the preservative and it did not inhibit the flow of the enzyme conjugate, wash or substrate fluids, thus it is doubtful that it altered test results.

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[3] Technical Manual. Biometallics, P.O. 2251, Princeton, NJ 08543

One reason for the large high number of high progesterone cows might be that the test did not reflect the true progesterone level since high fat milk was used. There appeared to be a disproportionate number of high progesterone samples in this study. Making the assumption that the milk fat was elevated and resulted in the increase in progesterone concentrations, it is possible that the test results were elevated by at least one level. Combining the two lowest readings (<2.0 ng/ml and 3.75 ng/ml) as indicative of estrus, the percentage of cows in estrus by farm were: Herd 1 = 52%, Herd 2 = 56%, Herd 3 = 89%, Herd 4 = 56%, Herd 5 = 56%, Herd 6 = 60%. This makes heat detection appear more successful but didn't change the conception rate by category. If high progesterone levels were associated with a low conception rate, a misidentification of cows in estrus would be suspected. However many cows became pregnant despite what appears to be high progesterone levels. Other authors have reported high progesterone levels at estrus (Bulman and Lamming, 1979; Pennington, et al., 1985; Marcus and Hackett, 1986).

Milk progesterone tests are reliable if used properly (Nebel et al., 1989). They can be a useful tool for the dairy producer and veterinarian in helping to manage reproductive processes in cattle. Other workers have projected the cost effectiveness for using milk progesterone assays (Pitcher and Galligan, 1990; Oltenacu, et al., 1990). These papers

described the effectiveness of using this test at 19-21 days after breeding to identify the non-pregnant animal. However, there is a need for a test which is sensitive enough to determine the low point in progesterone levels at estrus. This would assist the producer in making the decision of whether to breed the cow.

Milk progesterone testing as performed in this study did not prove to be useful in providing additional information to the dairy producer which would assist them in making a decision to breed a particular cow. The data shows that cows in which a high progesterone level was detected were just as likely to conceive as a cow in which a low level was found. A low reading would encourage the producer to inseminate the cow while a high reading really offered no information that the cow is not suitable for breeding at this time. The test used in this study was simple and convenient to use and had a distinct color change making it easy to interpret results. Additional work needs to be performed comparing premilk, composite milk and postmilk samples collected at estrus to determine if progesterone levels detected by this test would vary. Other available tests could also be compared to the test used in this study to determine if they were more accurate in their prediction of low versus high progesterone. Results of progesterone levels in milk samples tested by ELISA kits could be compared to RIA determination of progesterone levels in the samples to test for correlation.

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SUMMARY AND DISCUSSION

This study was conducted to determine if a commercially available milk progesterone ELISA test was sensitive enough to confirm estrus. Looking at 660 total milk samples from 6 dairies, the number of samples at each progesterone level were 235 at <2.0 ng/ml, 155 at 3.75 ng/ml, 115 at 5.5 ng/ml and 155 at >7.25 ng/ml. This is an uncharacteristic distribution since the cows were selected on the basis that they were in heat and suitable for breeding. When the progesterone levels were compared to the pregnancy or open status for each breeding there was no significant difference. In this study, high progesterone levels were just as likely to result in pregnancy as low levels. Some herds had better pregnancy rates at the high progesterone levels. Since sampling was done at breeding and breeding in the cow is usually performed near the end of or shortly after the end of estrus, progesterone levels should be low. Ovulation has not occurred at this time and there is no evidence to indicate luteinization of the follicle prior to ovulation in the cow. There are a few reported cases where other workers found elevated progesterone levels at breeding (Bulman and Lamming, 1979; Pennington, et al., 1985; Marcus and Hackett, 1986).

In looking at the conception rates between the herds significant differences were found between Herds 2 and 3 and between Herds 4 and 6. Herd 3 had the best percentage of

samples in the low progesterone group but its conception rate was not different from several of the other herds. This trial was interesting in that it appears that even in well managed dairies it is difficult to achieve conception rates over 50% if all breedings and cows are considered.

This study may have been flawed because samples were collected when it was most convenient for the sampler. Although the test instructions suggest the use of foremilk or composite milk, the manufacturer had indicated that there would most likely be no problem when using milk taken at the time of breeding. Since many dairymen breed cows after milking the assumption can be made that the sample more likely resembles postmilk which is higher in fat and therefore progesterone content. Other researchers indicate that milk with high fat content will interfere with progesterone binding and therefore the progesterone content will be determined to be lower than it really is. This work would appear not to confirm that statement. Since a disproportionately large number of samples in this study had medium to high levels of progesterone, it is assumed that the test detected the accurate level of progesterone. One other possibility for the high progesterone levels would be that the high fat content actually increased binding of antigen to antibody giving falsely elevated readings.

The test used in this study was simple and convenient to use and had a distinct color change making it easy to read

results. More work should be performed to determine which milk fraction should be used to obtain reliable results.

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