

Emerging Technologies in Bovine Pregnancy Diagnosis

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Introduction

Maintenance of milk production in the dairy industry depends upon parturition to initiate and restore lactation. Traditionally, it has been recommended that dairy cows maintain a 12 to 13 month calving interval for maximum productivity. Treatment with recombinant bovine somatotropin increases the persistency of lactation but long calving intervals increase the risk that a cow will be culled.

Early detection of pregnancy has been recommended to reduce the number of days open in cows that do not conceive after insemination. Pregnancy rates in modern dairy herds may be in the neighborhood of 35 to 40% (but are likely to be less). Thus, at least 60 to 65% of cows that are inseminated do not conceive and must be identified in order to be re-inseminated. In addition, the ability of managers to detect estrus appears to have deteriorated due to a number of factors that may include reduced intensity of estrus, increased herd size, and environmental factors. Difficulties in detecting estrus have resulted in the development and adoption of a number of schemes intended to manipulate ovarian function and inseminate cows by appointment without the need to detect estrus.¹⁻⁴

Reasons for pregnancy diagnosis: One of the most common procedures performed by veterinarians engaged in dairy practice is examination of cows and heifers to detect those that have been inseminated but have not conceived. Although there is some disagreement, most investigators agree that most profitable milk production results when the interval between parturitions is shorter rather than longer.⁵⁻⁸ Ideally, animals that were not pregnant would be identified within a few days after mating. They could be then treated with prostaglandin in the middle of the luteal phase of the estrous cycle and could then be inseminated again without further loss of time and production.

A number of methods have been used to identify non-pregnant cows. Perhaps the earliest method was observation for return to estrus. Animals that were not observed to return to estrus were assumed to be pregnant. Pregnancy is the most common cause for anestrus in dairy cows, but there are several pathological conditions that prevent return to estrus including pyometra, some ovarian cysts, and ovarian neoplasia. More importantly, the ability of managers to detect estrus on modern dairy farms is not adequate to detect estrus in many animals and failure to detect estrus accounts for much of the variation in days open between dairy herds.^{9,10}

Transrectal palpation of the uterus was first described in the eighteen-hundreds and has been the standard method since early in the last century.¹¹ The technique is thoroughly described by Zemjanis.¹² Although there has been some controversy regarding the interaction between pregnancy diagnosis by palpation and fetal loss, the procedure is probably safe when properly performed. Any pregnancy loss can be explained by factors that have a greater influence on calving rates than the embryonic age at pregnancy examination by transrectal palpation.¹³ More recently, electronic (ultrasound) and chemical tests for pregnancy have been developed. Several chemical tests have been, are, or probably will be commercially available and these are the subject of the majority of this paper.

Electronic Methods of Pregnancy Diagnosis

B-Mode Ultrasonography: The use of transrectal ultrasonography for detection of pregnancy in cows has been comprehensively described.¹⁴ While there is some variation among operators, image quality of the instrument, and animals, an accurate diagnosis of pregnancy can be made by approximately 26 to 28 days after ovulation when a 5 MHz transducer and a high-quality scanner are used.¹⁵ There are reports that pregnancy can be accurately detected earlier with 7.5 MHz transducers. Formulae for estimation of fetal age with ultrasonography have been published.¹⁴ The fetal heartbeat can be first detected at approximately day 21 and is the “gold standard” for proof of the presence of a viable conceptus.

Embryonic loss confounds pregnancy diagnosis by ultrasound as well as other methods of early pregnancy determination. Recent reports indicate that 10 to 16% of cows diagnosed pregnant early after insemination by ultrasound will undergo embryonic loss.¹⁶⁻²⁰ Therefore, a second examination at approximately 60 days after insemination to confirm pregnancy is required. Cows found non-pregnant at that time can be treated appropriately and returned to service without further loss of time.

Other Ultrasonic Instruments: Ultrasound scanners less expensive than the widely-used real-time scanners have been advertised from time to time but critical evaluations have shown them not sufficiently accurate for reliable detection of pregnancy.^{21,22}

Chemical Tests for Pregnancy

Progesterone. Luteal progesterone is required to maintain pregnancy in cows.²³⁻²⁵ However, concentrations of progesterone are elevated during the luteal phase of non-fertile estrous cycles; thus while the hormone is associated with pregnancy it is not a specific indicator of pregnancy.

One of the early chemical tests for pregnancy in cattle was measurement of concentrations of progesterone in milk or blood at 21 to 24 days after insemination. The basis for the test is the fact that if cows are pregnant, the corpus luteum persists and progesterone concentrations remain high, while if the animal is non-pregnant, luteolysis occurs at the end of the non-fertile cycle and progesterone concentrations are low. Progesterone assays were widely available both at central laboratories and as cow-side tests but the method has not been found to be sufficiently reliable and has largely fallen into disuse. Measurement of concentrations of progesterone in milk are reasonably accurate for identification of non-pregnant cows (94%) but not sufficiently accurate (77%) for detection of pregnant cows.²⁶ An attempt was made to differentiate between pregnant and non-pregnant cows by measuring plasma progesterone on days 4 and 8 after breeding but that application was not sufficiently accurate to identify cows that had failed to conceive.²⁷

Estrone Sulfate. Estrone sulfate, a conjugated estrogen, is the product of the fetoplacental unit and has been used to diagnose pregnancy in the cow and other species.^{28,29} Unfortunately, estrone sulfate concentrations in bovine maternal plasma are not sufficient to be detected until approximately day 72 after insemination, too late to be considered an early indicator of pregnancy.³⁰

Chorionic Gonadotropins. Aschheim and Zondek first reported the presence of a gonadotropic factor in the urine of pregnant women in 1927. Measurement of that

protein is the basis for modern laboratory tests for pregnancy in humans. This protein, now known as human chorionic gonadotropin (hCG), is a glycoprotein secreted by the trophoblast. A dimer consisting of alpha and beta subunits, it has a half-life of 8 hours due to its content of sialic acid. hCG is first detectable 8 to 11 days after conception and concentrations reach 50,000 U/L at 10 weeks and then decline to approximately 5,000 U/L at 14 weeks of pregnancy.³¹ Abnormal pregnancies are frequently characterized by an abnormal time course of hCG concentrations. In normal pregnancies, hCG doubles every 2-3 days during the first 6 weeks. A slower rate of increase is seen in cases of threatened abortion or ectopic pregnancy.³²

Another protein secreted by the human placenta is human placental lactogen (hPL), which is also called human chorionic somatotropin (hCS). hPL can be detected in the placenta 5 to 10 days after implantation but is not detected in maternal blood until 3 to 4 weeks later. hPL regulates lipid, carbohydrate, and protein metabolism.³³

The human placenta secretes many other pregnancy proteins. Pregnancy-specific B1 glycoprotein is secreted into the maternal circulation from the trophoblast but is also secreted by the gut and bone marrow. Pregnancy proteins are also over-expressed in many tumors of trophoblastic origin and in fetal abnormalities.³⁴

A protein with gonadotrophic activity in the serum of pregnant mares was demonstrated in 1930 by Cole and Heart.³⁵ First called pregnant mare serum gonadotropin (PMSG), this protein is now known as equine chorionic gonadotropin (eCG). eCG is secreted by the endometrial cups that are composed of trophoblastic cells with one or two nuclei that migrate to the maternal endometrium between days 36 and 38 after fertilization. After formation, the endometrial cups release eCG until approximately day 130 to 140 of pregnancy. eCG is the main luteotrophic factor in mares and its secretion coincides with the formation of secondary corpora lutea in the ovaries. The half-life of eCG in plasma is about 6 days due to its high sialic acid content. eCG is not secreted into the urine of pregnant mares.

Bovine chorionic somatomammotropin (bCS) or bovine placental lactogen (bPL) is produced by chorionic binucleate cells and is detectable in maternal serum by the fourth month of gestation. However, its concentration remains low (1 to 2 ng/ml) throughout gestation.^{36,37} Placental lactogens have been detected in pregnant ewes (ovine placental lactogen [oPL])³⁸ and in pregnant does (caprine placental lactogen [cPL]).³⁹

Early Pregnancy Factor/Early Conception Factor. Early pregnancy factor (EPF) is a protein that was first detected in the serum of pregnant mice within 4 to 6 hours after mating. EPF is composed of two components (EPF-A and EPF-B). EPF-A is secreted by the uterine tube and EPF-B by the ovary. Production of EPF-B requires a signal from the fertilized ovum (ovum factor). Ovum factor is released in the presence of prolactin after sperm penetration. EPF is an attractive marker for pregnancy in that it appears within hours after conception and disappears rapidly after death or removal of the embryo.⁴⁰⁻⁴²

Initially, EPF was detected by the rosette inhibition test. The assay is sensitive but time consuming and is not suitable for routine use.⁴³ More recently, a lateral flow dipstick was developed for detection of EPF as a “cow side” method to diagnose pregnancy. While an initial report indicated that the method was reliable to correctly diagnose non-pregnancy in 94.6% of cows at 24 to 48 hours after insemination,⁴⁴ more recent reports indicate that the cow-side test is not sufficiently accurate to be used as a management tool for dairy cattle.⁴⁵⁻⁴⁸ The manufacturer of the ECFTM recommends that

the test be used to identify non-pregnant cows.⁴⁹ It is recommended that milk samples be tested at 6 to 20 days after insemination and serum samples be tested 6 to 30 days after insemination. According to information supplied by the manufacturer, ECF becomes non-detectable in milk and serum at 20 and 30 days, respectively.

Maternal Recognition of Pregnancy

The process by which the dam recognizes the presence of an embryo varies among species and has been the subject of several recent reviews.^{50,51} Numerous signals are exchanged between dam and embryo to prevent luteal regression and maintain receptivity of the uterus to the presence of the embryo and its membranes. Detection of one (or more) of these signals could be a useful method of pregnancy detection because: 1) the protein (unlike progesterone) is a specific marker for pregnancy, and 2) the protein appears very early and failure to conceive could, in theory, be detected prior to the next anticipated ovulation.

In cattle and sheep, the embryo begins its efforts to prevent luteolysis prior to attachment to the endometrium. Large quantities of interferon-tau are released by the mononuclear cells of the trophoctoderm as the blastocyst begins to elongate on days 14-16 in cattle. Interferon-tau exerts its antiluteolytic effect by inhibiting endometrial expression of the oxytocin receptor through which circulating oxytocin stimulates episodic prostaglandin F₂alpha production.⁵²⁻⁵⁴ Interferon-tau would seem to be an excellent indicator of pregnancy since it is specifically associated with pregnancy and it is present prior to the next anticipated ovulation in non-pregnant cows. Unfortunately, interferon-tau remains within the uterine lumen and does not appear in measurable quantities in maternal blood or other body fluids.

After maternal recognition of pregnancy in ruminants, attachment to the endometrium begins. Invasiveness of the trophoblast is limited and the type of placentation (previously known as epitheliochorial and syndesmochorial) is now described as synepitheliochorial.⁵⁵ In cattle, areas of attachment are first observed at 20 days. Fetal binucleate cells migrate out of the trophoctoderm and fuse with maternal endometrial cells forming fetomaternal hybrid tissue. The binucleate cells are responsible for successful implantation and subsequent growth of the placentomes and produce and deliver protein and steroid hormones to the maternal circulation. Hormones are synthesized in binucleate cells and stored in granules. Fully granulated binucleate cells migrate to the fetomaternal syncytium. Granules are released by exocytosis into the maternal connective tissue.

Pregnancy-Associated Glycoproteins

Two pregnancy-specific proteins were isolated from bovine placenta by Butler et al (PSP-A and PSP-B).⁵⁶ PSP-A is not limited to pregnant animals but PSP-B was shown to be specific to the placenta. By use of a radioimmunoassay, Sasser et al were able to detect pregnancy from 24 days after conception.⁵⁷ Pregnancy diagnosis by assay of PBP-B is available commercially.⁵⁸ Serum samples are to be taken after 28 days post-insemination from heifers and after 30 days from lactating cows. Turn-around time for results is approximately 1 week. Measurement of PSP-B can also be used for detection of pregnancy in other ruminants including sheep, goats, mule deer, elk, red deer, moose, musk oxen, mountain goats, mountain sheep, and reindeer. Detection of PSP-B cannot

used to diagnose pregnancy in llamas, however.⁵⁹ Profiles of PSP-B concentrations, in conjunction with progesterone assays, have been used to attempt to measure the frequency of embryonic death in cows.⁶⁰ PSP-B has a long half and disappears slowly from the maternal circulation after parturition. The slow disappearance of PSP-B after calving may interfere with use of the test for diagnosis of pregnancy should blood samples for assay be taken too soon after calving.⁶¹ Interestingly, cows that are affected by retained fetal membranes and subsequent endometritis have been shown to have a higher concentration of PSP-B on the day of calving than their non-affected herd mates.⁶² PSP-B has been suggested to have a role in immunosuppression and the positive relationship between PSP-B concentration and retained placenta and endometritis may be a useful method to monitor postpartum health.

In 1991, Zoli et al, characterized a bovine pregnancy-associated glycoprotein (bPAG) from fetal cotyledons.⁶³ They subsequently developed a radioimmunoassay for diagnosis of pregnancy.⁶⁴ This molecule has since been designated bPAG-1 and later bPAG-I-67.⁶⁵ bPAG was detected in maternal serum at day 22 of pregnancy in some cows and by day 30 in all pregnant animals. Peak concentrations were found 1 to 5 days prior to parturition and became undetectable by 100 days postpartum. Detectable concentrations of bPAG were found in about 20% of unbred heifers and nonpregnant cows and in 15% of serum samples from bulls. bPAG1 and has been shown to be similar to if not identical to PSP-B.⁶⁶

The bPAG's are synthesized in a sub-population of binucleate cells in the trophoctoderm and stored in granules. As implantation is initiated, the binucleate cells migrate and fuse with the uterine epithelium. The secretory granules are released by exocytosis directly into the maternal circulation. The bPAG's belong to the family of aspartic proteinase family, some other members of which are pepsinogen and renin. Although their structure is similar, the bPAG's appear not to be active enzymes and their exact function is presently unknown.

It has been estimated that there are more than 100 bPAG's, many of which are expressed in the placenta.⁶⁷ These proteins appear, some for only a few days, at various times throughout gestation. Detection of these products of the placenta presents a unique opportunity for early and accurate detection of pregnancy. PAG's are not limited to species with synepitheliochorial placentas. Other members of the PAG family have been found in pigs,⁶⁸ horses,⁶⁹ and other species.⁷⁰

Most of the research conducted on the clinical application of detection of PAG's for diagnosis of pregnancy in cattle and other species has utilized radioimmunoassay systems to detect the proteins. This type of assay is not suitable for field use and must be conducted under controlled conditions where equipment and personnel suitable for utilization of radioactive isotopes are available. However, there is currently considerable interest in commercial development of "cow-side" assay systems that can be used for rapid and accurate identification of cows that are not pregnant. One such assay system that was under development when this manuscript was being prepared is the SurbredTM Rapid Pregnancy Test for Cattle.⁷¹ Information provided by the manufacturer indicates that two different tests are under development.⁷² Surbred 15TM is to be used to detect a specific antigen (unnamed) that appears in maternal blood between days 15 and 30 after insemination. Surbred 35TM is said to detect a different antigen (also unnamed) that is present between day 25 of gestation and term.

Application and acceptance of any test for pregnancy in cattle will depend upon a number of factors. Pitcher and Galligan⁸ evaluated the use of milk progesterone assays for diagnosis of pregnancy and listed the following inputs that affect the decision to use a test for diagnosis of pregnancy:

- cost of a day a cow is not pregnant
- conception rate
- cost of the test
- test sensitivity and specificity
- cost of insemination

Under conditions of their model, they determined that the value of the information yielded by early diagnosis of pregnancy was between \$6.00 and \$10.00 when conception rate was 75% and the cost of a non-pregnant day was \$2.00. Thomas Riley Marshall, former governor of the State of Indiana and 28th vice president of the United States (under President Woodrow Wilson) is famous for saying during a Senate debate, “What this country needs is a good five-cent cigar.” To paraphrase, perhaps what the dairy industry needs is a good under-\$6.00 pregnancy test.

Several effective protocols (Ovsynch, Cosynch, etc.) are available and are widely used in systematic breeding programs to insure timely first inseminations. Although re-synchronization protocols have been described to deliver second and subsequent inseminations in a timely manner, under most contemporary management conditions, many cows that do not conceive at the first insemination remain non-pregnant until at least 27 to 34 days (assuming weekly examinations) after breeding before they are identified by ultrasound or transrectal palpation of the reproductive tract. Once identified, these cows can then be re-enrolled in an estrus/ovulation synchronization protocol. Identification of non-pregnant cows earlier than 27 or 28 days would allow them to be returned to estrus/ovulation synchronization programs earlier and permit second (and third or fourth) inseminations when days in milk are fewer.

As noted above, early embryonic death is common during the first few weeks of pregnancy. Thus, cows could accurately be diagnosed as pregnant at 15 days after insemination, but could suffer embryonic death and return to estrus or be found non-pregnant when examined at a later stage of gestation. Thus, chemical tests such as those to detect PAG's, or any of a number of as-yet undiscovered markers for pregnancy, should properly be thought of as “tests for openness” rather than “tests for pregnancy”. If these tests are proven to be highly specific (proportion of animals detected that are truly non-pregnant), then the manager could be confident that the cow was indeed non-pregnant and could administer prostaglandin as part of a systematic breeding program with little concern that an undetected pregnancy would be terminated. However, since it can be assumed that a significant number of pregnancies would be spontaneously terminated (early embryonic death) after cows had been diagnosed pregnant, the sensitivity of the test may appear to be low. Thus, another factor that may influence a veterinarian's recommendation or a manager's decision to use a early pregnancy test is the necessity that cows diagnosed as “pregnant” during the first few weeks of gestation be re-examined at a later stage (perhaps 45 to 60 days) by some other method such as ultrasound or transrectal palpation to identify those cows that have lost their pregnancies during the interval between examinations.

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