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PREGNANCY DETERMINATION IN CATTLE: A REVIEW OF AVAILABLE ALTERNATIVES

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Introduction

Pregnancy testing in cattle has evolved over time. The simplest and most definitive test for pregnancy is to wait until the cow gives birth to the calf. This approach is perhaps acceptable for extensive systems but for intensive systems waiting until calving to identify the pregnant or nonpregnant (open) cows takes too long. The desire for an earlier pregnancy diagnosis led to the routine use of rectal palpation of the uterine contents for the purpose of detecting the pregnancy. Although traditionally practiced from 40 to 60 days after insemination or later, pregnancy diagnosis by rectal palpation can be pushed to its limit of detection (30 to 35 days after insemination) to identify open cows sooner. Additional sensitivity can be achieved by using transrectal ultrasound for pregnancy detection. Transrectal ultrasound can be used as early as 25 days after insemination but is more typically applied after day 30 (Fricke, 2002). If performed later (60 to 80 days) then the sex of the calf can be determined when ultrasound is used. Although ultrasound represents a definitive test for pregnancy and can be used to determine the sex of the calf, it requires specialized equipment and the examination generally requires more time than rectal palpation. Regardless of whether rectal palpation or ultrasound is used, an individual with highly specialized training performs the diagnosis. This individual is typically a veterinarian or, in some cases, may be a reproductive specialist that is an employee of the farm.

A changing cattle industry may affect how pregnancy diagnoses are performed in the future. Intensification of reproductive management in beef herds and the implementation of AI are creating the need for more accurate and timely diagnoses of pregnancy. At the same time, there is a shortage of large animal veterinarians in some regions (Jensen et al., 2009). The shortage of large animal veterinarians has put pressure on a limited number of experienced veterinarians to complete a large number of pregnancy diagnoses. In some cases there is the desire to perform the pregnancy exams sooner after insemination so that non-pregnant cattle can be identified earlier and resynchronized for a second AI. Collectively, these factors are creating an opportunity for the application of chemical pregnancy testing (for example, blood tests for pregnancy). Indeed, a recent report cited rapid growth in the application of one blood test for pregnancy (Figure 1). The cattle industry is clearly moving toward alternative methods of pregnancy diagnosis that do not require skilled practitioners or specialized equipment.

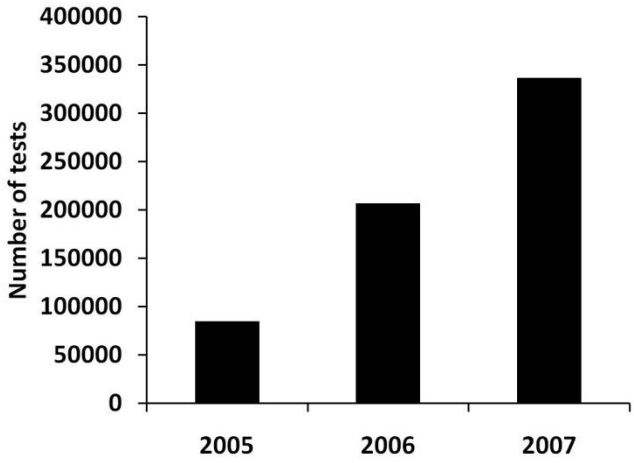

Figure 1. Number of BioPRYN® (BioTracking, LLC, Moscow, ID) tests performed in the years 2005, 2006, and 2007 (Stevenson, 2008). BioPRYN® is a commercial blood test for pregnancy in cattle.

Physiological and Theoretical Aspects of Four Tests

If cattle were people then the solution would be simple. The human pregnancy produces copious amount of a hormone called hCG (human chorionic gonadotropin) that passes into the urine and can be detected by a simple lateral flow ELISA test (Fletcher, 1986). This test is done by women in their homes. Unfortunately cows do not make bovine chorionic gonadotropin (or any such molecule that is readily detectable in the urine) so a simple test that is similar to the human test is not available. There are, however, a series of candidate molecules associated with pregnancy in cattle (Figure 2). These molecules include: "early pregnancy factor", interferon-stimulated genes (ISGs), progesterone, and pregnancy-associated glycoproteins (PAGs).

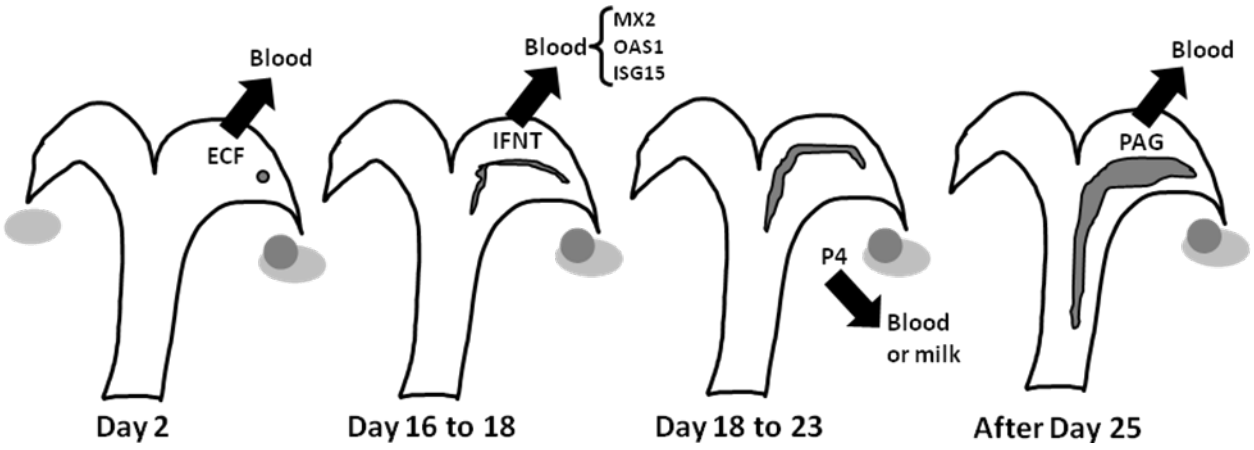

Figure 2. Four chemical tests for pregnancy. Pregnancy can be detected at different intervals after insemination by measuring different chemicals in the blood. In this figure, the uterus, embryo (grey structure within the uterine horn), and ovary (ovoid structure with circular corpus luteum) are depicted. The ECF test (left-most depiction) was reported accurate on day 2 but the test was later shown to be inaccurate at any time. Other tests measure the biological response to interferon-τ (day 16 to 18), progesterone in blood or milk (day 18 to 23), or pregnancy associated glycoproteins (PAG; after day 25). See text for details on the individual tests.

Early Pregnancy Factor

A chemical test for pregnancy termed Early Pregnancy Factor (EPF) or Early Conception Factor (ECF) was proposed and marketed in the 1990's (Concepto Diagnostics, Knoxville,TN). This molecule was supposedly present in the blood of pregnant cattle within two days after conception. The exact nature of this molecule (an immunosuppressive glycoprotein protein) and how it got into circulation were not well defined but nonetheless it could be assayed by using a rosette inhibition test (Nancarrow et al., 1981). A kit for pregnancy diagnosis reached the market but three different studies found that the kit was unreliable for pregnancy diagnosis (Cordoba et al., 2001; Gandy et al., 2001; Ambrose et al., 2007).

The possibility of performing an early pregnancy diagnosis (within one day after insemination) is, of course, intriguing in as much as it may be possible to resynchronize open cows within one week after insemination. To our knowledge, very little is known about the secretions of the early bovine embryo (within one week after conception). Detection of these secretions in blood, milk, or urine for the purpose of pregnancy diagnosis is an interesting area for investigation. There would theoretically be a large amount of embryonic loss after the diagnosis but the truly nonpregnant cow could be dealt with in a timely manner (within one week after insemination). If the cow were synchronized for first insemination and not pregnant then the second insemination could be done after a luteolytic dose of $PGF_{2\alpha}$ given at least one week after first insemination.

Interferon-stimulated Gene Expression

The early embryo forms a blastocyst and hatches out of the zona pellucida at approximately one week after fertilization. During the second week, it continues to grow, becomes spherical, and during the third week elongates to form the filamentous embryo. It is during this transition from the spherical to elongated form that the embryo produces interferon-τ (INFT). The INFT is produced in large amounts by the embryo after day 14 to signal the mother and establish the pregnancy (Roberts, 2007). The INFT secretion is transitory. It reaches a maximum by 20 to 24 days and is completely gone by day 30 of pregnancy. The IFNT is unlike hCG because its expression is transitory and it does not accumulate in the blood or urine. Thus, IFNT cannot be used for a pregnancy test in the blood or urine of the cow.

Although the INFT cannot be assayed directly in blood, its presence can be detected through its action on leukocytes (white blood cells). Interferon-τ is a cytokine, a class of molecules that has the capacity to stimulate leukocyte function. The leukocyte response to INFT can be monitored by measuring the expression of secondary proteins that are called "interferon-stimulated genes" (ISGs) within leukocytes (Gifford et al., 2007). Examples of ISGs are MX2, ISG15 and OAS1. An example of the ISG response is shown in Figure 3 (data for MX2). In this experiment, dairy cows were blood sampled on days 14, 16, 18, and 20 after insemination and were later diagnosed as either pregnant or open by ultrasound. The RNA from the leukocytes was extracted from the blood and analysed by using a process called "reverse-transcriptase PCR" (RTPCR). The graph shows that the expression of MX2 increases in the leukocytes of pregnant cows particularly on days 18 and 20. This increase in the MX2 represents the leukocytes responding to the IFNT produced by the embryo.

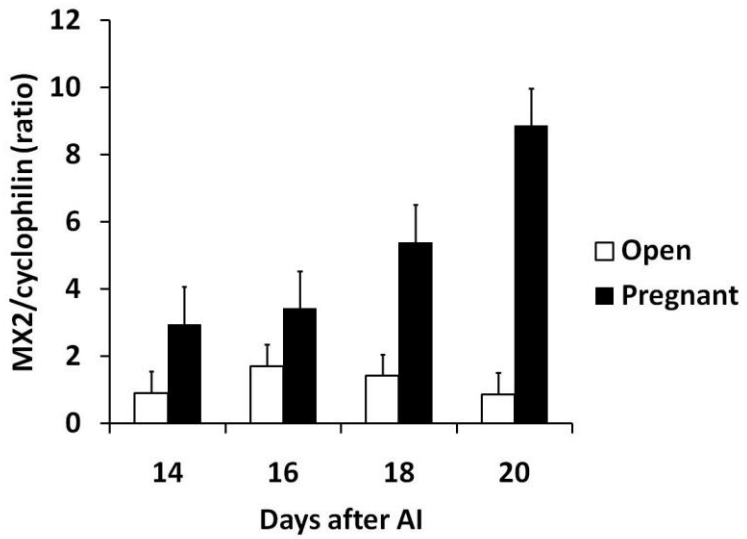

Figure 3. Ratio of MX2 to cyclophilin gene expression in leukocytes isolated from cows on days 14, 16, 18, and 20 after insemination. Compared with open (nonpregnant) cows, the pregnant cows have an increase in the ratio. The increase in ratio represents the stimulatory effect of interferon-τ from the embryo on the expression of MX2 in the leukocytes. The increase in MX2 relative to cyclophilin (control gene) can be used as an indication or pregnancy.

Despite their promise as a diagnostic tool for pregnancy detection, the ISGs have not seen commercial application. The current and most reliable assay method involves RNA extraction and RTPCR. The RNA in leukocytes is a highly labile molecule and blood samples would theoretically require special handling before analysis. The RTPCR is a cumbersome and time-consuming laboratory process (relative to ELISA, for example). Although individual cows may give a strong signal by day 18, our experience is that a consistent signal is not achieved until day 20 after insemination. This is only five days earlier than the simpler and perhaps more reliable PAG test (see below). A breakthrough in this area may come if the protein instead of the RNA for ISGs could be measured in blood or if IFNT itself could be measured in blood. Blood proteins are typically more stable than blood RNA and may be more suitable for on-farm applications.

Progesterone Monitoring

Measuring progesterone in blood or milk as a method to identify open (nonpregnant) cows was perhaps the first true example of chemical pregnancy testing. If a cow is not pregnant then she will theoretcially have a decrease in progresterone at approximately 21 days after insemination. If she is pregnant then her progesterone concentrations will remain elevated (Figure 4). There is excellent physiological underpinning for the progesterone test because cows cannot be pregnant if they have low (less than 1 ng/mL) progesterone 21 days after insemination.

Several studies examined progesteorne testing after insemination (reviewed by Nebel, 1988). The test can be done on the farm (milk progesterone test for dairy cows) or in the laboratory. When done on the farm and with a single sample (for example on day 21) progesterone testing is an excellent method for identifying a truly open cow. If a cows tests low for progesterone then she is not pregnant (progesterone testing has a high negative predictive value). If a cow tests high for progesterone then she *may be* pregnant (progesterone has a low postive predictive value). The origins of the low positive predictive value are illustrated in Figure 4. In theory (left hand panel), all inseminated cows are properly synchronized, have low progesterone at insemination and an

increase in progesterone after insemination. Twenty-one days later when the test is done, pregnant cows (A) have elevelated progesterone and open cows have low progesterone because they have returned to estrus (B). In the theoretical case, the progesterone test can properly identify both statuses because the progesterone concentrations are very different (A versus B). In reality (right hand panel, Figure 4), cows at 21 days after insemination are a mixed population. Some cows are pregnant (A) and some cows are clearly not pregnant (B, C, D, and E). Among the cows that are not pregnant, are the cows that are returning to estrus normally (B), cows that have a delayed return to estrus (C; this is generally caused by embryonic loss between day 18 and 24), cows that were never synchronized properly in the first place (D; high progesterone at AI) and anovular cows (E). The open cows that are in categories C and D test positive for high progesterone and contribute to the lower positive predictive value of the test.

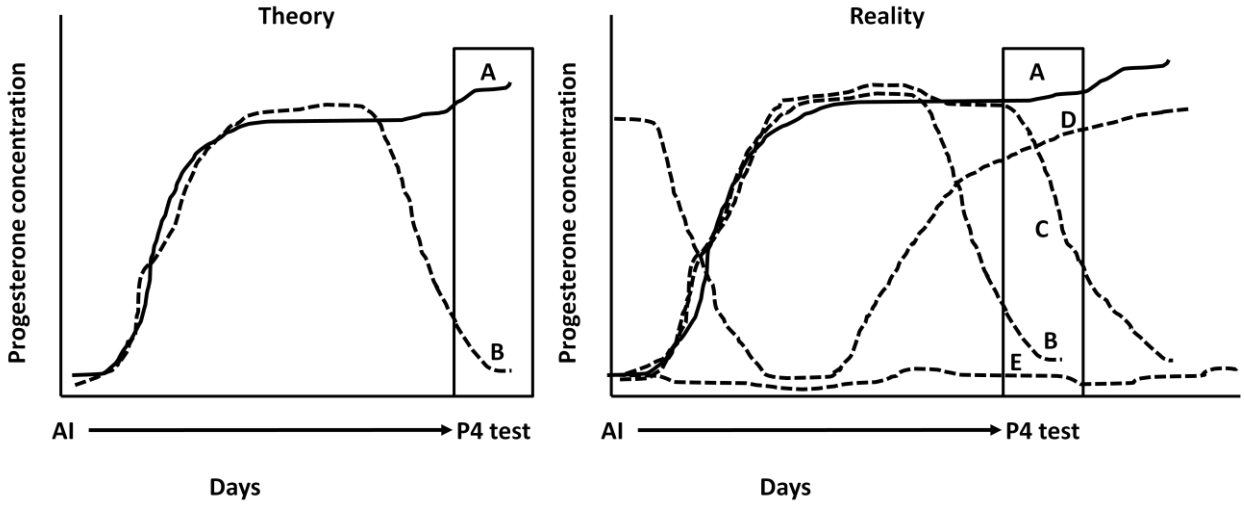

Figure 4. Progesteone concentration in inseminated cattle and the application of a progesteorne test. In the theoretical case (left) a cow is inseminated (AI) and progesterone increases. Pregnant cattle have a sustained increase in progesterone (A) and nonpregnat cattle undergo a decrease in progesterone (luteolysis; B). A progesterone test is based on these differences in progesterone (P4 test; box). In reality (right graph) cows may be pregnant (A), nonpregnant with normal luteolysis (B), nonpregnant with delayed luteolysis (C), not synchronized for AI correctly (D) or anovular (E). The mixture of cows at the time of the P4 test (box) makes interpretation of the results difficult. The low positive predictive value that arises from the issues raised in the right hand panel (Reality) cannot easily be resolved if a single measurement for progesterone is made. It is unlikely that single tube assays for progesterone could be completed on multiple days for muliple cows on a farm. Progesterone testing, therefore, has limited applicability for pregnancy diagnosis.

Pregnancy-associated Glycoproteins (PAGs)

The binucleate cells of the embryonic trophectoderm (placenta) migrate and fuse with the epithelial lining of the uterus (Spencer et al., 2007). The binucleate cells are unique because they secrete placental proteins into the capillary beds of the uterus that connect to the peripheral circulation of the cow. One type of protein secreted by the binucleate cells is the pregnancy-associated glycoprotein (PAG). The PAGs consist of a large family of more than 20 closely related proteins that are only produced by the placenta (Telugu et al., 2009). They can be detected in the blood of pregnant cows beginning at approximately 25 days after insemination (Green et al., 2005). This family of proteins is expanding in the bovine genome but their function

is unknown. Monitoring the concentrations of PAGs in blood is an effective method of pregnancy detection.

The original work on PAGs was done by Sasser et al. (1989) in which they described proteins in the blood of pregnant cows. The protein that they isolated was called "pregnancy-specific protein B" or PSPB. The PSPB is a member of the PAG family of proteins produced by the placenta. From a biochemical perspective, PSPB is the same thing as boPAG-1 (Green et al., 2005). The PAGs are in some respects similar to hCG, the basis of the human pregnancy test. Both hCG and PAG are placental glycoproteins with long half-lives in blood. The two proteins have, however, entirely different molecular structures and functions. Both can be readily detected in blood but to our knowledge, PAG, unlike hCG, cannot be detected in urine.

The original PAG (PSBP) test is commercially available through BioTracking, LLC (Moscow, ID) or through one of the commercial labs affiliated with BioTracking (located throughout the United States). The test is trade-named "BioPRYN" and if the sample is shipped to BioTracking then the charge is $2.40 per sample for standard overnight assay service or $3.50 per sample for same-day assay service. The PSBP protein is stable in blood. Blood samples are collected from cattle that are at least 30 days after insemination and shipped at room temperature. Data (pregnancy status of individual animals) are returned to the producer via telephone, mail, fax, or email. The test has an extremely high negative predictive value (99.9%; data provided on company website). The high negative predictive value means that if a cow is diagnosed open than she is definitely open. The positive predictive value is also extremely high (approximately 95%) but slightly lower than the negative predictive value. The slightly lower positive predictive value is caused by a small percentage of pregnant cattle that undergo embryonic loss after testing. Pregnant cattle that undergo embryonic loss will initially test positive (pregnant) but will later be found open because the embryo died (Figure 5). The PAGs from the previous pregnancy are found within the blood stream for several months after calving. It is necessary, therefore, to wait at least 90 d postpartum before testing a cow for pregnancy when the standard BioPRYN® test is used (Figure 5). Newer versions of the BioBRYN® test may enable testing of earlier postpartum cows. Users, therefore, should consult BioTracking about the earliest possible postpartum test date for cows. Virgin heifers can be tested at any time because a positive result cannot be confused with a previous pregnancy.

In addition to the BioPRYN test there are now two new commercially available PAG tests. Conception Animal Reproduction Technologies (Beaumont, Quebec, Canada) has partnered with AgSource Cooperative Services and Genex Cooperative (Cooperative Resources International. CRI, Shawano, WI) to market a test called DG29. The test can be conducted on cows or heifers 29 days or more after insemination. Cows must also be at least 90 days after calving before blood samples are collected. The second new commercially available test is being marketed by IDEXX laboratories (Westbrook, ME). The test can be used after 28 days of pregnancy and results are available within 2.5 hours. Cows that are 60 days or greater postpartum can be tested.

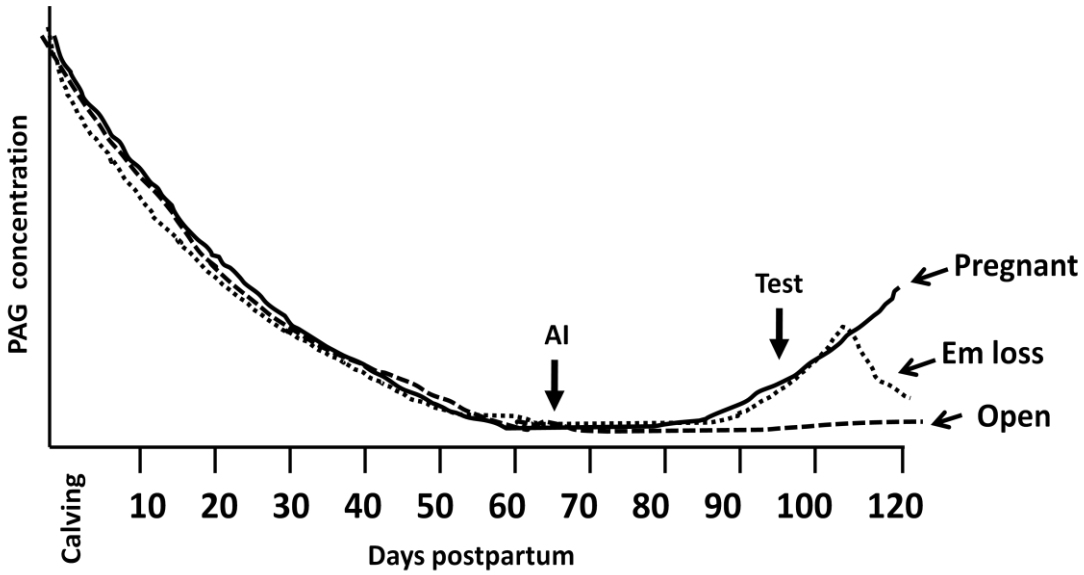

Figure 5. Conceptual diagram of the blood PAG concentrations in postpartum cattle. The PAGs are placental proteins that are secreted into the blood where they have a long half-life. At calving, the blood PAG concentrations are extremely high. For boPAG-1 (PSPB), 90 d are required for the PAG from the previous pregnancy to entirely clear the blood stream. Once cleared, blood PAG concentration can be used as a pregnancy test. In this example, the AI is 65 days postpartum and cows are tested at 95 days. Pregnant cows have elevated PAG whereas open cows do not. A cow that undergoes embryonic loss (Em loss) will have an increase in PAG until the embryo dies. The PAG will decrease in blood after the embryo dies and will require 1 to 2 weeks to clear from the blood stream.

Practical considerations for PAG testing. The advent of reliable and affordable PAG assays for pregnancy testing in cattle creates options for early pregnancy diagnosis (28 to 30 days after insemination). Transrectal ultrasonography can be used within approximately the same time frame as the PAG test (Fricke, 2002). There are advantages of ultrasonography when compared with the PAG blood test. For example, ultrasound provides an instantaneous diagnosis of pregnancy and the ability to evaluate uterine and ovarian morphology of nonpregnant animals. It may also be possible to identify dead embryos and nonviable pregnancies when the ultrasound is used. These advantages must be weighed against the cost of ultrasound equipment, the technical skill required when performing the ultrasound procedure, and whether or not the farm has access to someone who can do early pregnancy diagnosis with ultrasound (Fricke, 2002).

Integrating Pregnancy Tests into a Reproductive Program

Pregnancy diagnosis from a blood sample enables the detection of nonpregnant (open) cows sooner after insemination. At this time, commercially available blood PAG tests can be performed at 28 days after insemination and PAG tests that are in development may push this interval to 25 days. In all likelihood, future tests for pregnancy will decrease further the interval between insemination and pregnancy detection. For example, if the ISGs are used then an interval as short as 18 to 20 days may be achievable (Figure 2). Shortening the interval between insemination and pregnancy detection also enables a shorter interval between successive inseminations for herds performing synchronization and resynchronization without estrus detection.

The downside of earlier pregnancy diagnosis is that some cows diagnosed pregnant will later be found open because pregnancies are lost over time when embryos die. Most cows are pregnant shortly after insemination but there are sequential periods of embryonic loss until the end of pregnancy. Most of the embryonic loss occurs before the placenta is fully formed at approximately 60 days after insemination (Santos et al., 2004). If cows are checked too early then a high percentage of the cows diagnosed as pregnant will later be found open because they have lost their pregnancies through the natural process of embryonic loss (Figure 6). If the cows are checked too late then open cows go undiagnosed for too long. In these open cows, earlier pregnancy detection could enable corrective intervention (for example resynchronization) so that they have an additional opportunity for a pregnancy to AI.

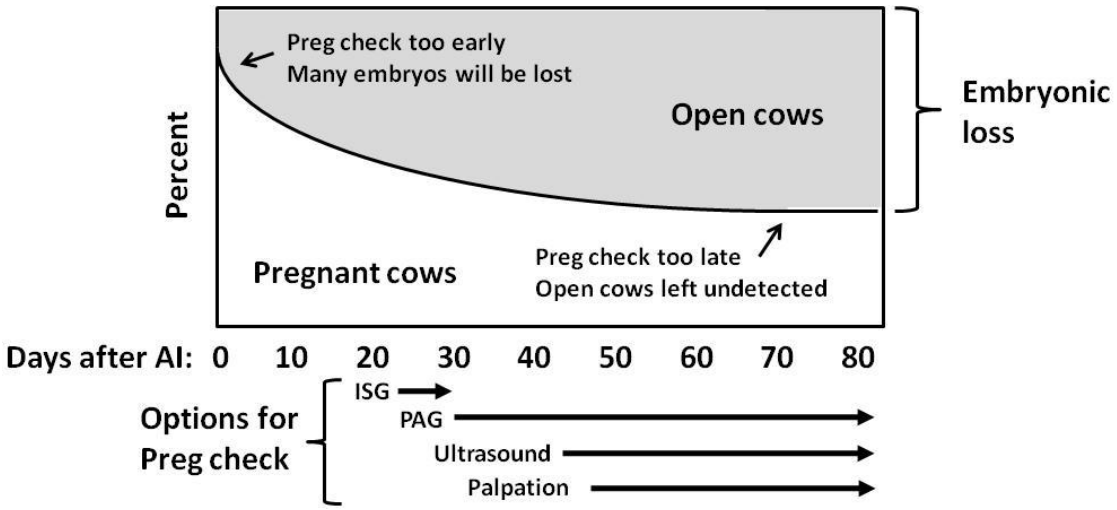

Figure 6. Conceptual diagram showing important considerations when making a decision about when to perform pregnancy diagnosis after an insemination (AI). Cows undergo embryonic loss after they conceive. A high percentage of cows are pregnant soon after insemination (top panel, left part of graph). The percentage of pregnant cows decreases over time because of the natural process of embryonic loss. If cows are pregnancy diagnosed ("Preg checked") too early then many cows that are diagnosed as "pregnant" will lose their pregnancies during the embryonic loss period. The losses may diminish the value of the early diagnosis. If the preg check is scheduled too late (after most of the loss is completed) then an open cow may not be identified until too late in the breeding season.

Conclusions

Cattlemen have options for pregnancy diagnosis. The traditional method of manual palpation is widely practiced. In some areas, ultrasound is performed so that more information is collected and pregnancies are detected sooner after insemination. In some geographical regions, there are too few skilled practitioners that can perform pregnancy diagnosis by manual palpation or ultrasound. In these places, blood pregnancy tests for PAGs are a viable option for pregnancy diagnosis that can be used at any time after 30 days of pregnancy. The PAG tests are based on well-understood physiology and are commercially available from at least three suppliers at competitive prices. Newer blood tests for PAG may enable pregnancy detection as early as 25 days after insemination. There is also the possibility of ISG tests that could determine pregnancy

by 18 to 20 days after insemination. If cows are checked too early, however, then a high percentage of the cows that are diagnosed as pregnant will later be found open because they have lost their pregnancies through the natural process of embryonic loss. An appropriate method for pregnancy diagnosis depends on the objectives of the reproductive program and considerations that are unique to each individual farm.

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