Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows

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Abstract

ISG15 is induced by conceptus-derived interferon-τ in the endometrium on days 15–45 of pregnancy. It was hypothesized that pregnancy induces blood cell ISG15 gene expression and that low blood ISG15 mRNA levels provide an indication of non-pregnant cows on day 18. Blood was collected either on day 18 (n = 78) or on days 15–21, 25, and 32 (n = 21; serial collection) from dairy cows following artificial insemination (AI). Plasma progesterone concentration was determined using RIA. ISG15 mRNA levels were determined using real-time PCR. Pregnancy was diagnosed on day 32 using transrectal ultrasound. ISG15 mRNA levels increased after day 16, peaked at day 20 and then declined to day 16 levels by 32 days following AI. The average pregnancy rate was 43% based on blood cell ISG15 mRNA. The average pregnancy rate was 33% based on the transrectal ultrasound. Lower levels of ISG15 mRNA or progesterone during serial collections were 100% accurate in predicting non-pregnant cows based on day 32 transrectal ultrasound. However, detection of ISG15 mRNA yielded 78% accuracy in predicting pregnant cows, while progesterone yielded 58% accuracy. Average plasma progesterone based on pregnancy status according to ultrasound was consistently higher in pregnant (> 4 ng/ml) when compared with non-pregnant cows from days 15 to 32, except on day 16. It is concluded that detection of low blood ISG15 mRNA levels during serial collection from days 17 to 25 serves as an accurate indicator of cows that are not pregnant, thus allowing re-synchronization and insemination.

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Introduction

Improved management, nutrition, and genetic selection have dramatically changed the dairy industry in the United States. However, increased milk production per cow has negative effects on reproductive efficiency by increasing the incidence of infertility (Lucy 2001). Reproductive efficiency could be improved with early detection of pregnancy following artificial insemination (AI). Early detection of pregnancy would allow producers to identify non-pregnant cattle and to synchronize and artificially inseminate these cows prior to the next ovulation.

Two methods are presently used for pregnancy diagnosis in cattle. One approach is through palpation per rectum for the presence of the positive signs of pregnancy, which is useful in detecting embryos after 40–50 days of pregnancy (Beal et al. 1992, Fricke 2002). Pregnancy can be determined as early as 35 days using palpation per rectum, but this is not recommended because manipulation of the uterus and the fetal membranes can lead to abortion (Abbitt et al. 1978, Vaillancourt et al. 1979). The second most implemented method of pregnancy diagnosis is transrectal ultrasound, which is accurate as early as day 27 of pregnancy, but also requires a skilled technician (Beal et al. 1992, Fricke 2002). Both methods are relatively late following maternal recognition of pregnancy, which occurs between days 14 and 18 in the cow (Bazer et al. 1991, Thatcher et al. 1995).

Other peripheral indicators of early pregnancy in the cow include blood levels of early pregnancy factor (EPF; Cordoba et al. 2001, Gandy et al. 2001) and pregnancy-specific protein B (PSPB; Austin et al. 1999). Use of EPF, also referred to as early conception factor, as a diagnostic for early pregnancy has been problematic (Cordoba et al. 2001, Gandy et al. 2001). PSPB, also known as pregnancy-associated glycoprotein-1 (Roberts et al. 1995), is accurate in predicting pregnancy after day 30 (Szenic et al. 1998). Pregnancy-specific protein B has an exceptionally long half-life and remains in circulation for several months following parturition, affecting the PSBP as a marker for pregnancy when cows are inseminated prior to 70 days post partum (Kiracofe et al. 1993, Austin et al. 1999).

Detection of the steroid hormone, progesterone, can also be used to determine pregnancy status in cows (Dobson & Fitzpatrick 1976, Macfarlane et al. 1977, Booth et al. 1979). Since most estrous cycles are 16–24 days in length with an average of 21 days, it is possible to sample most non-pregnant cows at a time that progesterone concentration is low, which
makes them distinguishable from pregnant cows that have elevated progesterone concentration. However, because non-pregnant cows may also have elevated progesterone, use in predicting pregnancy is more accurate after days 21–24 post-AI.

The conceptus (embryo proper and trophoblast) releases signals to maintain production of progesterone by the corpus luteum during early pregnancy (Spencer & Bazer 2002, Thatcher et al. 2005). During the period of maternal recognition of pregnancy in cows (days 15–18), the conceptus is free-floating in the uterus. Interferon (IFN)-τ is a major paracrine signal that is produced by the bovine conceptus and acts on the endometrium to elicit secondary responses that are necessary to maintain pregnancy (Roberts et al. 1992, Thatcher et al. 1995, Spencer & Bazer 2002). Conceptus-derived IFN-τ disrupts the signal transduction pathway that regulates release of prostaglandin F2α (PGF), which is the major luteolytic product in cattle (Thatcher et al. 1995, 2001). IFN-τ also induces synthesis and secretion of a ubiquitin homolog (ubiquitin cross-reactive protein) that is called ISG15 (as described by HUGO gene nomenclature). ISG15 is induced in the uterus by pregnancy in cows (Austin et al. 1996, Hansen et al. 1997, Johnson et al. 1998, Perry et al. 1999, Thatcher et al. 2001), mice (Austin et al. 2003) and primates (Bebington et al. 1999a,b). While it has been shown to be induced by IFN-τ in ruminants, the cytokine or growth factor that induces ISG15 in mice and primates has not been described.

Bovine ISG15 is released by the endometrium at times that are coincident with IFN-τ release from the conceptus (Austin et al. 1996, Hansen et al. 1997, 1999, Johnson et al. 1998). Likewise, ISG15 is found in significant amounts in uterine flushings from day 18 pregnant cows. Since ISG15 functions as an intracellular ubiquitin homolog (Haa et al. 1987, Loeb & Haas 1992, D’Cunha et al. 1996a,b, Johnson et al. 1998) and as an extracellular cytokine (Recht et al. 1991, D’Cunha et al. 1996a,b), we suspected that it might serve as a peripheral surrogate marker for early pregnancy in ruminants.

Development of an early diagnostic for pregnancy would help in managing cows that failed to have viable embryos on day 18 following insemination. For example, many dairy cows must be re-inseminated after transrectal ultrasound or PSPB diagnosis on day 30 because they are not pregnant after first insemination. Identification of non-pregnant cows on days 18–20 after first insemination would facilitate a second insemination of non-pregnant cows approximately 10 days earlier than waiting for transrectal ultrasound or PSPB diagnosis and would reduce associated economic losses (Lucy 2001). We hypothesized that ISG15 mRNA would be greater in blood from day 16 to 23 of pregnancy when compared with non-pregnant cows using semi-quantitative real-time PCR approaches. We also hypothesized that low levels of ISG15 mRNA would be an accurate indicator of cows that were not pregnant. Finally, we suspected that intermediate or delayed increases in blood ISG15 levels would reflect cows that were carrying embryos that are destined to die.

Materials and Methods

Animal care and blood collection

Investigations were conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and were approved by the University of Wyoming Animal Care and Use Committee (OPRR-PHS A-3216-01). Mr B E Chapman from the Diamond D Dairy (Mead, Colorado, USA) kindly provided AI records, transrectal ultrasound results (approximately day 32) and cows that were used in this study. Estrous cycles were synchronized using GnRH-based OvSynch protocol by injecting GnRH (Cystorellin, 100 μg/cow, i.m.) on day-10, followed by an injection of PGF (Lutalyse, 25 mg/cow, i.m.) on day-3 followed by an injection (i.m.) of GnRH (100 μg/cow) on day 1. Cows were artificially inseminated on day 0. Blood was collected either on day 18 of pregnancy (day 18; n = 78; includes day 18 during the serial collection) or on days 15, 16, 17, 18, 19, 20, 21, 25, and 32 of pregnancy (serial collection; n = 21) following AI. Blood was collected in Vacutainers containing potassium EDTA from the caudal tail blood vessels and 200 μl was mixed with 750 μl TRI reagent (Sigma) and stored at −80 °C until analysis. The remaining blood sample was chilled and plasma was separated through centrifugation for progesterone analysis. Pregnancy was determined on day 32 using transrectal ultrasound.

RNA extraction and cDNA synthesis

RNA was extracted from 200 μl whole blood using TRI reagent BD. Total RNA was treated with DNase I, followed by phenol:chloroform:isoamylalcohol extraction. Single-stranded cDNA was synthesized from total cellular RNA (equal volumes) using iScript Reverse transcriptase (Bio–Rad) at 25 °C/5 min, 42 °C/30 min, and then 85 °C/5 min. Product was diluted 5X with DNase/ RNase-free water.

Semi-quantitative real-time PCR

Diluted cDNA (10 μl) was used as a template for semi-quantitative real-time PCR amplification using SYBR Green (Bio–Rad). glyceraldehyde 3’ phosphate dehydrogenase (GAPDH) National center for biotechnology information (NCBI) Accession no.: BC102589) was used as an internal reference for normalization of ISG15 mRNA (NCBI Accession no.: NM_174366) expression. Bovine ISG15 (forward; 5’GGTATCCGAGCTGAAGCAGTTT3’, reverse; 5’ACCTCCCAGTGCAGGT3’) and GAPDH (forward; 5’GAGTTGTCAGCAAATGCTCCT3’, reverse; 5’GGTCTAAGAGGCTCCACGAGA3’) primers were designed to generate an amplicon size of 87 and 94 bp respectively. c-PCR for ISG15 and GAPDH cDNA amplification was performed using 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 40 cycles (Bio–Rad iCycler, Bio–Rad). Following RT-PCR, cDNAs were melted (melting
curve) to ensure the quality of amplification. For melting curve analysis, RT-PCR products were incubated for 10 s at each step with an increase in temperature by 0.5 °C from 55 to 95 °C in each cycle. The RT-PCR product was resolved as a single band using agarose gel electrophoresis, purified and submitted for sequencing to confirm identity with ISG15. ISG15 mRNA expression level was reported relative to GAPDH. Prediction of pregnancy based on blood ISG15 mRNA level was determined using −7.0 as the arbitrary threshold level relative to GAPDH expression on or after day 18 post-AI. For instance, cows with ISG15 mRNA levels (relative to GAPDH) over −7.0 were considered to be pregnant, while those with mRNA levels under −7.0 were considered to be non-pregnant. This arbitrary threshold was chosen by comparing the profiles of ISG15 mRNA levels over several days prior to disclosure of transrectal ultrasound results. This level of ISG15 mRNA was sustained over 4 or more consecutive days beginning on day 18 post-AI.

**Progesterone analysis**

Plasma progesterone concentration was determined by solid-phase RIA (Diagnostics Products Corporation, Los Angeles, CA, USA) as described by Eggleston et al. (1990). The antibody against progesterone had <2.5% cross-reactivity with related compounds as reported by the manufacturer. Standards were prepared in charcoal-stripped steer serum. The assay sensitivity was 15.6 pg/tube. Inter- and intra-assay coefficients of variation were <7.3%. Animals with progesterone levels >2.0 ng/ml were predicted to be pregnant, while those with progesterone levels <2.0 ng/ml were considered to be non-pregnant.

**Statistical analysis**

The blind experiment was designed as follows. Transrectal ultrasound results (Dr Kevin McSweeney, DVM for Double Diamond Dairy, Mead, Colorado, USA) were kept confidential until pregnancy status determined by ISG15 mRNA data were submitted to Dr Mark Colgin (AspenBio, Inc; Castle Rock, CO, USA). Prediction of pregnancy by blood ISG15 mRNA was determined by ISG15 mRNA gene expression relative to GAPDH over the arbitrary level of −7.0 on day 18 after AI (day 18) or for 4 consecutive days following 18 days after AI (serial collection). Pregnancy prediction using plasma progesterone was determined when progesterone level was >2.0 ng/ml.

Predictions of pregnancy status using ISG15 mRNA and progesterone levels in blood were then compared with the transrectal ultrasound data. The results of each experiment were presented as correct pregnant (TP), incorrect pregnant (FP), correct non-pregnant (TN), and incorrect non-pregnant (FN). Sensitivity (probability that diagnosis is pregnant among cows, which are truly pregnant; TP/(TP + FN) × 100), specificity (probability that diagnosis is non-pregnant among cows, which are truly non-pregnant; TN/(TN + FP) × 100), positive predictive value (PPV), (probability that the cow is truly pregnant if the diagnosis is pregnant; TP/(TP + FP) × 100) and negative predictive value (NPV): (probability that the cow is truly non-pregnant if the diagnosis is non-pregnant; TN/(TN + FN) × 100) were calculated.

A general linear model (GLM) was used to examine effects of day of pregnancy on expression of ISG15 mRNA and progesterone in repeated measurements. Least square means were considered different when P<0.05. Differences in pregnancy determination were evaluated using chi-square analysis of SAS.

**Results**

**Day 18 and 32 of pregnancy**

Transrectal ultrasound on day 32 post-AI revealed pregnancy rates that were typical of most dairy operations with 33% found to be pregnant and 67% found to be non-pregnant. Average pregnancy rate, predicted by ISG15 blood mRNA (43%) and tested using χ² analysis, was not different from average pregnancy rate (33%) based on transrectal ultrasound. The 43% pregnancy rate, based on elevated blood ISG15 mRNA levels, was probably due to presence of a conceptus, and signaling for ISG15 mRNA on day 18. On day 18 only, ISG15 mRNA was 89% accurate in NPV, while progesterone was 100% accurate in NPV. However, progesterone was 47% accurate in PPV, while ISG15 mRNA was 62% accurate.

**Serial bloods**

In the serial collection, both low ISG15 mRNA and low progesterone profiles were 100% accurate in predicting non-pregnant cows as non-pregnant (NPV; Table 1). However, high ISG15 mRNA resulted in detecting fewer (P<0.05) incorrect pregnant cows when compared with plasma progesterone (2 vs 5 cows). Positive prediction value was 78% based on blood cell ISG15 mRNA and 58% based on plasma progesterone concentration. This means that 22% of the time, pregnancy prediction failed when using ISG15 mRNA as an indicator, whereas pregnancy prediction using progesterone as an indicator failed 42% of the time.

**Average plasma progesterone**

Average plasma progesterone concentration was greater (P<0.05) in pregnant cows when compared with non-pregnant cows on day 18 (3.63±0.19 vs 2.67±0.23 ng/ml) and when averaged for days of serial collection (4.22±0.12 vs 2.29±0.14 ng/ml). Plasma progesterone in the serial collection experiment was averaged across the cows based on pregnancy status determined by transrectal ultrasound (Fig. 1). Average progesterone concentration was consistently high in pregnant cows and was maintained at concentrations that were >4.0 ng/ml from days 15 to 32, except on day 16.
Average progesterone concentration in non-pregnant cows was below 3.0 ng/ml and the pattern followed normal progesterone levels indicative of non-pregnant cycling cows with progesterone declining through day 21 and then increasing after day 21.

Plasma progesterone profiles followed five distinctly different profiles (Fig. 2) as did ISG15 mRNA levels. First, progesterone was consistently low (<2.0 ng/ml) from day 15 to 32 (A; n = 1). Second, progesterone was low prior to day 21, but then increased to day 32 (B; n = 3). Third, progesterone was high on day 15 and remained high through day 25, but then declined to low levels by day 32 (C; n = 4). Fourth, in pregnant cows, progesterone concentration was >4.0 ng/ml and was maintained at this level from 15 to 32 days of pregnancy (D; n = 8). Fifth, in non-pregnant but cycling cows, progesterone levels decreased from day 18 and then increased at day 32 after AI (E; n = 5).

**ISG15 mRNA**

Blood cell ISG15 mRNA also was averaged and presented based on pregnancy status determined by transrectal ultrasound (Fig. 1). ISG15 mRNA expression in the blood increased after day 16, peaked at day 20 and then declined to day 16 levels by 32 days following AI. Note that ISG15 mRNA levels remained above the arbitrary cycle threshold level of −7.0 from day 18 through day 25 of pregnancy. ISG15 mRNA in blood from non-pregnant cows was variable, but remained below the threshold level of −7.0 for this experiment. The overall mean ISG15 mRNA expression in the blood from days 15 to 32 was 4.6-fold greater (P<0.05) in pregnant when compared with non-pregnant cows.

ISG15 mRNA profiles in blood when compared with progesterone concentrations are shown in Fig. 2. In the case where progesterone was low throughout the sampling period, ISG15 mRNA was also low (<−7.0) and there appeared to be a decline in ISG15 mRNA from day 15 through 18 followed by an increase from day 18 to day 21 and a decrease from day 21 to 32 (A; n = 1). In the second scenario, blood ISG15 mRNA levels

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**Table 1** Prediction of pregnancy by ISG15 blood mRNA or progesterone in comparison with ultrasound (day 32) from dairy cows

<table>
<thead>
<tr>
<th></th>
<th>Serial collection (ISG15)</th>
<th>Serial collection (progesterone)</th>
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<td>100</td>
</tr>
</tbody>
</table>

^aSensitivity: probability that diagnosis is pregnant among cows which are truly pregnant; correct pregnant/correct pregnant + incorrect non-pregnant×100.
^bSpecificity: probability that diagnosis is non-pregnant among cows which are truly non-pregnant; correct non-pregnant/correct non-pregnant + incorrect pregnant×100.
^cPPV (positive predictive value): probability that the cow is truly pregnant if the diagnosis is pregnant; correct pregnant/correct pregnant + incorrect pregnant×100.
^dNPV (negative predictive value): probability that the cow is truly non-pregnant if the diagnosis is non-pregnant; correct non-pregnant/correct non-pregnant + incorrect non-pregnant×100.

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![Figure 1](https://example.com/fig1.png) Average plasma progesterone and relative expression of blood ISG15 mRNA of pregnant (pregnant) or non-pregnant (open) cows from day 15 through day 32 after artificial insemination. Average plasma progesterone of pregnant animals was maintained at over 4 ng/ml, except at day 16, while open animals showed typical progesterone pattern of non-pregnant, cycling animals. Average whole blood ISG15 mRNA from pregnant animals remained at greater levels than non-pregnant animals (P<0.05) with the greatest level of expression occurring at day 20 post-AI.
Figure 2  Plasma progesterone (left hand panels) and whole blood ISG15 mRNA profiles (right hand panels) of dairy cows from day 15 through day 32 after artificial insemination. (A; \(n = 1\)) This animal is non-pregnant, non-cycling. (B; \(n = 3\)) These animals did not conceive and are cycling. (C; \(n = 4\)) These animals may have had an embryo which died prior to day 32. (D; \(n = 8\)) These animals are pregnant as evidenced by high levels of progesterone and ISG15 mRNA from day 15 through 32 post-AI. (E; \(n = 5\)) These animals are non-pregnant, cycling animals.

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were consistently $<-7.0$, which is reflective in this study of a non-pregnant cow ($B; n = 3$). Third, ISG15 mRNA was low during early establishment of pregnancy (15–18 days), but then remained elevated ($>-7.0$) from day 19 to 25, followed by a severe drop ($<-25$) in ISG15 by day 32 ($C; n = 4$). The pregnant cows had ISG15 levels that were consistently $>-7.0$ from day 15 through 32 of pregnancy ($D; n = 8$). Finally, non-pregnant-cycling cows had ISG15 mRNA levels that remained below $-7.0$ except on day 25 ($E; n = 5$).

In the present study, five cows were misdiagnosed as being pregnant using plasma progesterone levels as a determinant. Of the false positives, two cows were also predicted to be pregnant based on blood ISG15 mRNA levels. Of the remaining three cows, two cows had blood ISG15 mRNA profiles that were similar to a pregnant cow, but the level of ISG15 did not increase above the threshold that was set for pregnant cows in this analysis. The last cow had ISG15 mRNA levels that varied over days examined and this variation was interpreted to reflect a non-pregnant cow, while diagnosis based on progesterone concentration resulted in a false positive.

**Discussion**

One major constraint to optimal production in beef and dairy industries is the inability to identify non-pregnant cows early after breeding (Lucy 2001). This delays the decision to rebreeding cows as much as 30 days (time when transrectal ultrasound is accurate by an experienced veterinarian). Development of an early diagnostic for open cows would help producers make a decision on how to manage the non-pregnant cow. For example, a diagnostic, which accurately distinguishes non-pregnant cows on day 18 after breeding, would allow the producer to synchronize and artificially inseminate these cows prior to the next ovulation.

Another constraint to beef and dairy industries is the incidence of early embryo mortality (Thatcher et al. 2001, 2005). Death of the embryo is a serious economic loss and is caused by a dysfunctional communication between the mother and the embryo. Exactly when and why embryo mortality occurs is the subject of study in our laboratory. A long-term goal of these experiments is not only to be able to identify non-pregnant cows, but also to predict pregnant cows that are destined to loose the embryo. Examination of plasma progesterone levels coupled with determination of blood cell ISG15 mRNA levels that are profiled over several days provides an excellent overview of corpus luteum function and presence of a viable embryo.

Yankey et al. (2001) reported that peripheral blood mononuclear cells become activated by conceptus-derived IFN-τ and express higher levels of MX mRNA in pregnant when compared with non-pregnant sheep. It has been known for several years that ISG15 is expressed in greater levels in the endometrium from pregnant when compared with non-pregnant cows (Austin et al. 1996, 2004, Johnson et al. 1998). ISG15 also functions as an extracellular cytokine (Deblandre et al. 1995, D'Cunha et al. 1996a,b, Pru et al. 2000) and can be found in media from cultured endometrium and uterine flushings (Austin et al. 1996). Study of IFN-induced gene expression in white blood cells from early pregnant cows has not been studied until the present experiments. In the present experiments, we tested the hypothesis that ISG15 mRNA was upregulated in blood from pregnant when compared with non-pregnant cows.

When examining average plasma progesterone concentration and average blood ISG15 mRNA levels, both were significantly greater in pregnant when compared with non-pregnant cows on days 17, 18, 19, and 20, and 25. Day 18 was selected for more extensive examination of blood cell ISG15 mRNA expression, because it represented a time during which the average ISG15 mRNA levels were significantly different between pregnant and non-pregnant cows. It was also late enough following AI for establishment of pregnancy in cows with viable embryos. Finally, it was early enough in a normal estrous cycle to prepare for re-insemination within 21 days following first service. Predicted non-pregnant cows based on low blood ISG15 was 100% accurate when examining the serial collection. However, prediction of non-pregnant cows on day 18 based on low blood ISG15 mRNA levels was problematic when considering the negative predictive value. ISG15 mRNA was 89% accurate in predicting correct non-pregnant cows when examined on a single day, day 18, following AI. Accuracy might be affected by the nature of semi-quantitative SYBR Green RT-PCR. More quantitative approaches such as multi-channel detection of ISG15 and GAPDH mRNAs (e.g. multiplexing) might help in improving diagnosis of open cows on day 18. Progesterone in single or serial blood collections had high sensitivity and negative predictive value in the present study. This is an important practical issue, since tests with a high negative predictive value will avoid injecting pregnant females with luteolytic agents, thus avoiding potential iatrogenic abortion.

Blood is composed of different types of cells, which include erythrocytes, immature reticulocytes, thrombocytes, as well as granulocytes, lymphocytes and monocytes. Variable expression of ISG15 mRNA in these cells may also contribute to variable ISG15 mRNA expression and false diagnosis of pregnancy based on high ISG15 mRNA levels. Examination of ISG15 mRNA in sub-populations of isolated peripheral blood mononuclear cells may improve the accuracy of pregnancy determination and may reduce the false negative rate when compared to examining whole blood mRNA pools.

The variability in ISG15 mRNA levels in bloods on day 18 might also be caused by a slight delay or differences in amount of IFN-τ released by the conceptus due to variations in development during this period. Variations in blood ISG15 mRNA levels could also be caused by diminished IFN-τ release that is caused by embryo mortality. Regardless, examination of ISG15 mRNA levels in blood in the serial collection experiment (days 15–32) was 100% accurate in predicting...
non-pregnant cows. Because of this result, we expect that a single determination on day 19, 20, or 21 might be as accurate in predicting non-pregnant cows as the serial collection experiment. We are presently determining which day(s) provides the best blood ISG15 indication of a non-pregnant cow and of a cow that has an embryo that will survive.

Use of blood ISG15 mRNA as an early indicator of cows that are carrying viable embryos that survive the embryonic period is problematic. Only 62% of pregnant cows were diagnosed correctly based on ISG15 mRNA levels on day 18 and only 78% of pregnant cows were diagnosed correctly based on ISG15 mRNA in the serial collection. This is in contrast to the higher false positive rates when using progesterone as an indicator of pregnancy in the day 18 (47% diagnosed correctly) and serial (58% diagnosed correctly) collections.

In order to better understand the relationships between ISG15 and pregnancy status, plasma progesterone was also examined in the serial collection and was used in combination with ISG15 to classify individual cows in one of five classifications. Based on these classifications, data were grouped to provide means, which were then analyzed.

The single cow that was not cycling had low plasma progesterone during the entire serial collection period. The lack of an increase in progesterone following AI, with an increase in progesterone from days 21 to 25 was interpreted to mean that ovulation and formation of a corpus luteum did not occur following AI, but ovulation and formation of a corpus luteum did occur 21 days later. High levels of progesterone from days 15 to 18, followed by a decline by day 19 and then an increase by day 32 was representative of cows that had ovulated, failed to conceive to AI, but then ovulated again about 21 days later and had a functional corpus luteum by day 32. In each of these non-pregnant scenarios, average ISG15 mRNA levels were highly variable, but remained below the threshold level for designation as a pregnant cow.

Cows with high plasma progesterone concentrations from days 15 through 32 and with ISG15 levels above the threshold level from day 17 through day 25 were interpreted to be pregnant and to have contained a viable embryo that survived through day 32. This was also supported by the presence of an embryo on day 32 based on transrectal ultrasound. One interesting finding in this study was the detection of cows undergoing embryo mortality using this approach. For example, four cows had high plasma progesterone levels from days 15 to 21 followed by a decline in progesterone on day 25 to levels indicative of luteolysis by day 32 following AI. ISG15 mRNA levels reached the threshold value for pregnant cows on day 19, but this increase was delayed when compared with the increase in ISG15 mRNA by day 17 in the pregnant cows. The other observation which is interpreted to reflect embryo mortality is the abrupt decline in ISG15 mRNA from day 21 to day 25 and day 32. The delayed increase in blood ISG15 during establishment of pregnancy followed by the decline in ISG15 is hypothesized to reflect a conceptus that was delayed or diminished in IFN-τ signaling. The decline in ISG15 from days 21 to 32 was associated with a decline in progesterone to sub-luteal levels and was reflective of an embryo that had failed to fully initiate the anti-luteolytic mechanism and had died. Transrectal ultrasound on day 32 confirmed the lack of an embryo in these cows.

ISG15 is one of several IFN-induced genes that are activated in response to viral infection (Nakaya et al. 2001, MacQuillan et al. 2003, Lenschow et al. 2005). Thus, in cases of viral infection, diagnosis of pregnancy using ISG15 may be complicated. In the case that viral infection is detected by high ISG15 mRNA levels in the blood, it could be argued that the false positive rate would increase. Cows suggested to be pregnant based on high ISG15 mRNA could be re-tested later during pregnancy on day 32 using transrectal ultrasound to confirm pregnancy status. In the case that there was no embryo present, more specific diagnostics could be done to determine the nature and possible long-term health consequences of the viral infection.

Virally infected cows with greater ISG15 mRNA or protein levels would have no consequences in identifying uninfected non-pregnant cows, which would be the primary use of this technology. The other finding from these studies is the ability to identify cows undergoing embryo mortality from those that have a viable embryo. It may become possible to more clearly delineate the causes of embryo mortality by focusing on IFN-τ release and the uterine response. Does the embryo die because of corpus luteum insufficiency, a lack of endometrial and blood cell response to IFN-τ, a delayed or diminished release of IFN-τ or a combination of all of these critical responses to pregnancy? We are presently studying these relationships using blood cell ISG15 mRNA expression and plasma progesterone concentrations as markers of a viable embryo.

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