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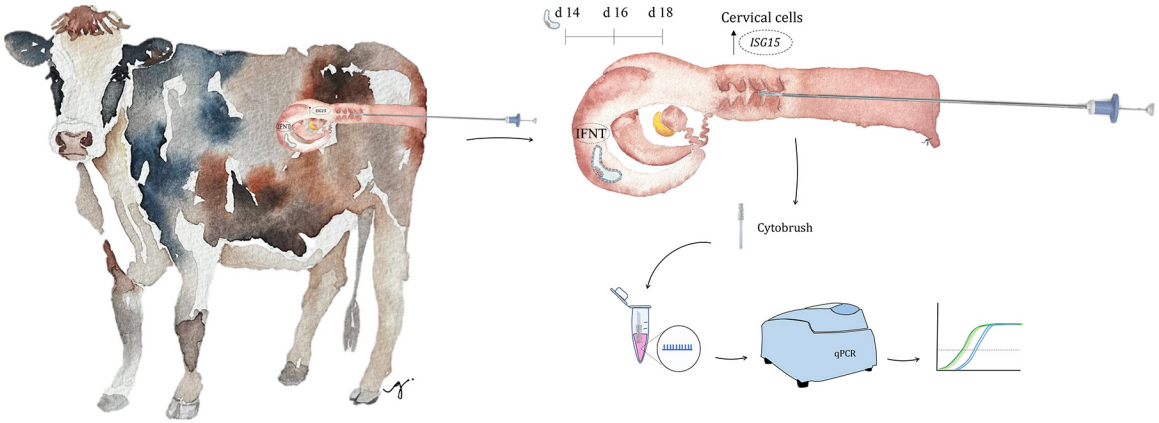
Short Communication
Physiology

Increased expression of interferon-stimulated gene 15 (*ISG15*) in cervical cells on day 14 of pregnancy in Holstein heifers

Graciana R. Mendina,[1]* Victoria de Brun,[2] Maria de Lourdes Adrien,[1] Victoria Pons,[1] Rodrigo Vivian Paradizo,[3] Jorge Gil,[1] Cecilia C. Rocha,[4] Mario Binelli,[4] and Ana Meikle[2]

Graphical Abstract

Increased cervical *ISG15* mRNA expression from day 14 to 18 of pregnancy in Holstein heifers



Summary

In cattle, the ability to diagnose pregnancy status before the natural return to estrus allows for the detection of early embryo losses and the use of resynchronization programs for rebreeding in a short period of time. The production of interferon tau (IFNT) by trophoblastic cells starts in the early embryo stages. Expression of IFNT-stimulated genes in the cervix has been reported as an early pregnancy diagnostic tool as early as day 17 of pregnancy. We compared the expression of *ISG15* in cervical cells between pregnant and cyclic heifers (control, sham-inseminated) on days 14, 16, and 18 after insemination. The expression levels of *ISG15* in cervical cells were significantly greater in pregnant compared with control heifers on day 14, and remained greater on days 16 and 18. A receiver operator characteristic (ROC) curve analysis showed the most accurate prediction of pregnancy on day 16.

Highlights

- Pregnant heifers had greater *ISG15* expression in cervical cells as early as day 14 compared with controls.
- Cervical *ISG15* expression was greater on day 16 than on day 14 in pregnant heifers.
- A ROC curve analysis showed the most accurate prediction of pregnancy on day 16.

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Increased expression of interferon-stimulated gene 15 (*ISG15*) in cervical cells on day 14 of pregnancy in Holstein heifers

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Abstract: In cattle, expression of IFN-stimulated genes in the female reproductive tract has been reported as an early pregnancy diagnostic tool, as early as d 17 of pregnancy. The hypothesis of this study was that expression of *ISG15* in the cervix of pregnant heifers is increased on d 14 of pregnancy. The objective was to compare the expression of *ISG15* in cervical cells between pregnant and cyclic heifers (control, sham-inseminated) on d 14, 16, and 18 after insemination (d 0). Holstein heifers were submitted to an estrus synchronization protocol and inseminated with extender only ("control," n = 6), or with regular semen (n = 15). Heifers were classified as pregnant (n = 10) by ultrasound at d 30 through the detection of a viable embryo with a heartbeat. Blood samples from the coccygeal vein were collected to determine serum progesterone concentrations on d 14, 16, and 18. The expression of *ISG15* and *PGR* in cervical cells collected through cytobrush was measured on d 14, 16, and 18. A receiver operating characteristic (ROC) curve was calculated to quantify the pregnancy diagnostic accuracy of *ISG15* and *PGR* expression. The expression levels of *ISG15* in cervical cells were significantly greater in pregnant compared with control heifers on d 14, and remained greater on d 16 and 18, whereas differences in PGR were observed only on d 18. Scatter plots and ROC analyses showed the most accurate prediction of pregnancy for *ISG15* on d 16. In conclusion, cervical cells express greater *ISG15* mRNA in pregnant versus control heifers as early as d 14 postinsemination, with the best accuracy on d 16.

The ability to diagnose the pregnancy status before the natural return to estrus allows the detection of early embryo losses and the use of resynchronization programs for rebreeding in a short period (Motta et al., 2020). The capacity to reduce the time to conception and improve reproductive efficiency increases the profitability of dairy (Inchaisri et al., 2010) and beef (Lamb and Mercadante, 2016) cattle production systems. However, the search for an early pregnancy biomarker has yielded limited results.

To achieve a successful pregnancy, the semi-allogeneic conceptus produces a trophoblast-derived cytokine called interferon tau (**IFNT**) to induce immunological tolerance toward itself and prevent luteolysis (reviewed by Forde and Lonergan, 2017; Talukder et al., 2020). The production of the IFNT starts as early as d 7 (Sponchiado et al., 2017; Rashid et al., 2018), reaching a peak in its expression on d 15 to 16 of pregnancy (Farin et al., 1990). Since the discovery that IFNT triggers the expression of interferon-stimulated genes (**ISG**) in the bovine endometrium (Naivar et al., 1995), many researchers sought to use them as early pregnancy markers in a variety of tissues and cell types.

Greater expression of ISG has been found in pregnant cows' white blood cells mostly between d 16 and 20 (Gifford et al., 2007; Pugliesi et al., 2014; Haq et al., 2016; Sheikh et al., 2018; Melo et al., 2020b), liver on d 18 (Meyerholz et al., 2016), corpus luteum on d 16 (Yang et al., 2010), endometrium from d 15 (Austin et al., 2004; Forde et al., 2011; Moraes et al., 2020; Adhikari et al., 2022), and cervical and vaginal cells from d 17 to 18 (Kunii et al., 2018; Ferraz et al., 2021; Domingues et al., 2024). Moreover, greater *ISG15* mRNA expression was detected as early as d 7 postinsemination only in the uterotubal junction of the uterine horn ipsilateral to the CL of pregnant cows, but not in other regions of the uterine horns, suggesting that at this early stage of development, closer proximity with the embryo is necessary to stimulate the expression of ISG (Sponchiado et al., 2017). However, sampling the cranial endometrium to measure ISG expression as an early biomarker of pregnancy is impracticable to the maintenance of the pregnancy.

Recently, researchers found increased expression of *ISG15* as early as d 17 in cervical and vaginal cells of lactating Holstein cows (Kunii et al., 2018; Domingues et al., 2024). Interestingly, the cervical expression of *ISG15* was around 16-fold greater than the traditional methodology of measurement in blood immune cells (Kunii et al., 2018; Domingues et al., 2024). Nevertheless, in these studies, *ISG15* was detected in pregnant versus nonpregnant cows, without comparing to a cyclic noninseminated group, which is necessary to establish a basal threshold of *ISG15* expression for pregnancy diagnosis.

It is well known that progesterone (**P4**) modifies the reproductive tract physiology for pregnancy maintenance. Although it is accepted that in ruminants, in vivo implantation events are preceded by the loss of expression of progesterone receptor (*PGR*) in the luminal epithelium (Bazer et al., 2008), it has been also shown in

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in vitro bovine endometrial cells that IFNT induces the expression of *PGR* and that there are IFN genomic binding sites for IFN-responsive factors in the *PGR* gene (Palma-Vera and Einspanier, 2016). Overall, less information is available on *PGR* in the cervix, and we have not found reports on cervical *PGR* expression during early pregnancy.

Thus, as there is evidence that IFNT is produced even before the maternal recognition of pregnancy (i.e., d 16) and that it induces the expression of ISG in surrounding reproductive tissues, we hypothesize that pregnant heifers have greater expression of *ISG15* in cervical cells as early as d 14 postinsemination compared with control sham-inseminated heifers. The aim of this study was to compare the gene expression of *ISG15* and *PGR* on d 14, 16, and 18 postinsemination in the cervix of pregnant and control Holstein heifers.

Cyclic Holstein heifers between 15 and 18 mo of age and weighing 360 ± 36 kg, maintained at the Experimental Station Dr. Mario A. Cassinoni, Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay (32° 23′07.6″ S 58° 03′17.9″ W), were used for this study. The research protocol was approved by the Ethics Committee of Universidad de la República, CEUA-CHEA ID 14/2023-Exp. 311170–000129–23). The experiment was performed from October to November of 2023. Heifers were kept in grazing paddocks (*Lolium multiflorum* and *Trifolium pratense*), and received supplementation (1.6 and 0.8 kg/animal per day of soybean hulls and ground corn, respectively) and water ad libitum. Heifers were submitted to an estrus synchronization protocol based on 2 injections of 2 mL of $PGF_{2\alpha}$ analog ($PGF_{2\alpha}$; D-cloprostenol, 0.075 mg/mL, Ciclar, Zoovet, Argentina), i.m., 11 d apart. In both injections, heifers were fitted with estrus detection patches (Estrotect, Rockway Inc., USA). Visual estrus detection was conducted 3 times a day by a single experienced operator, starting 12 h after the second $PGF_{2\alpha}$ injection, and continued for 96 h. Heifers were considered in estrus when standing to be mounted or when more than 50% of the silver coating of the patch was removed. Heifers were distributed randomly in 2 treatments: controls, sham-inseminated with semen extender only (n = 6), and inseminated with regular semen (n = 15) from a single Holstein bull previously used in the experimental station herd with satisfactory results. A pool of 2 straws of the same batch used in the experiment was analyzed for kinematics by the computer-assisted semen analyzer (CASA) system, as well as subsequent morphological evaluation. Animals were inseminated between 6 and 12 h after the first visualized standing estrus event or patch indication. Blood samples were taken from the coccygeal vein using an evacuated tube system (Vacuette 8 mL Serum Beads Clot Activator, Greiner Bio-One GmbH) on d 14, 16, and 18 after insemination. Blood samples were centrifuged at 1,680 × *g* for 10 min at room temperature and serum was stored at −20°C until further analysis. Epithelial cervical cell samples were obtained by a single experienced operator, blinded to group assignment, using a cytological brush (Cytobrush, Sakira S.A., China) coupled to the tip of a conventional AI gun, covered by a disposable AI sheath and protected by a sanitary sheath, as described previously by Cardoso et al. (2017), on d 14, 16, and 18 after insemination. The apparatus was inserted via the cervix and rotated to harvest cells from the cervical canal near the external os of the cervix. The cytobrush was uncoupled from the apparatus and placed into a 2-mL cryotube filled with 1 mL of Trizol reagent (Life Technologies) for mRNA extraction and stored immediately in liquid nitrogen at −196°C. Samples were then stored at −80°C until mRNA extraction. Confirmatory pregnancy diagnosis was performed on d 30 postinsemination using transrectal ultrasonography with a linear transducer (Aloka 500, 7.5 MHz, Tokyo, Japan) through the detection of a viable embryo with a heartbeat. Animals were then classified as pregnant (n = 10) and nonpregnant. Nonpregnant heifers were excluded from the analysis because of the uncertain outcome of their pregnancies, which may have included embryo loss at different stages, associated with transient increases in IFNT of varying magnitudes. A subsequent diagnosis was performed on d 90, confirming that 4 heifers did not maintain pregnancy after d 30; however, due the limited number of animals no further analysis was performed other than control versus pregnant heifers on d 30. Serum P4 was determined on d 14, 16, and 18 postinsemination by a solid-phase RIA using a commercial kit (MP Biomedicals, Los Angeles, CA) as reported by Ruprechter et al. (2020). All samples were analyzed in a single assay; the sensitivity was 0.11 ng/mL; the intra-assay CV was 3.2%. The RNA extraction was performed using Trizol according to the manufacturer's instructions, as reported by Fernández-Foren et al. (2023). The concentration and purity of the RNA were determined using a spectrophotometer (NanoDrop ND 1000; Thermo Scientific, Wilmington, DE). Total RNA was treated with DNase using a DNA-free kit (Ambion, Austin, TX). For each sample, cDNA was synthesized by reverse transcription using a SuperScript III transcriptase (Invitrogen) with random primers and 1 µg of total RNA as a template. Real-time PCR (**qPCR**) was performed using a SYBR Green master mix (Thermo Fisher Scientific) and samples were analyzed in a Rotor-Gene 6000 kit (Corbett Life Sciences, Sydney, Australia). The efficiency of the assay was calculated according to Rutledge and Côté (2003). Sequences, the expected product lengths, and efficiency of primers to amplify cDNA of the target genes *ISG15*, progesterone receptor (*PGR*), and the endogenous control β-actin (*ACTB*) were as follows: *ISG15* (NM_001009735.1) forward: GGTATCCGAGCTGAAGCAGTT, reverse: ACCTCCCTGCTGTCAAGGT, 87-bp amplicon, efficiency: 2.04; *PGR* (NM_001205356.1) forward: GACAGCACTTTCTAGGCGACAT, reverse: TGTGCTGGAAGAAACGATTGC, 79-bp, efficiency 2.10; *ACTB* (BT030480) forward: CGTGGCTACAGCTTCACC, reverse: GAAATCGTCCGTGACATCAA, 53-bp, efficiency 1.94. Gene expression was measured by relative quantification to the calibrator (pool of mRNA from each sample enrolled in the study, analyzed in duplicate) and normalized to the endogenous control gene (*ACTB*) using the Pfaffl method, considering the respective amplification efficiencies (Pfaffl, 2001). Sample size calculations were performed using Proc Power (SAS Studio, SAS Institute Inc., Cary, NC). Serum P4 concentrations and gene expression were analyzed by a Glimmix procedure (Proc Glimmix; SAS Studio) for repeated measures having as a basic unit the heifer nested into group, with an autoregressive order 1 correlation structure. Fixed effects included the group, day, and the interaction between group and day. Gene expression data did not follow normal distribution in the Shapiro-Wilk test ($\alpha < 0.05$) and were transformed to natural logarithms. The transformed data were used to calculate *P*-values, whereas the corresponding untransformed LSM and SE are reported for clarity. Significance was considered with $\alpha \leq 0.05$, and tendency between 0.05 and 0.10. According to the differences in the mRNA relative expression between groups, a receiver operating characteristic (**ROC**) curve for the *ISG15* expression on each day and *PGR* on d 18 was generated

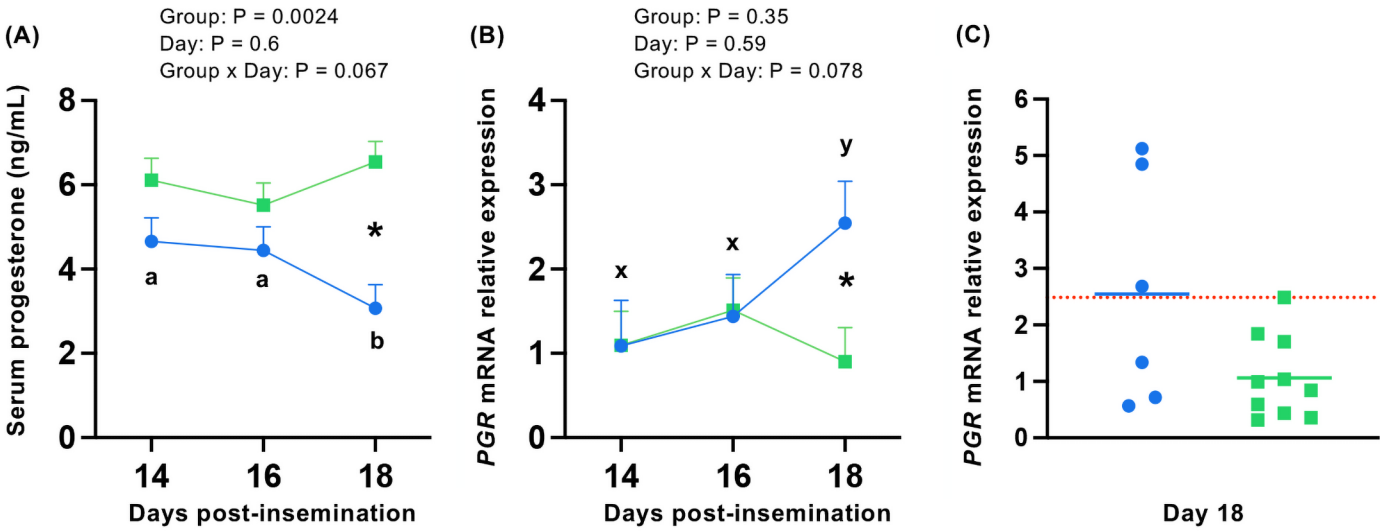

Figure 1. Serum progesterone concentrations (A) and relative mRNA expression of *PGR* (B) on d 14, 16, and 18 postinsemination, and scatter plot with individual distribution of *PGR* relative mRNA expression on d 18 (C) in control (blue) and pregnant (green) heifers. Dots show the individual values. Means are indicated by the continuous midlines. Red dotted lines indicate the threshold for pregnancy diagnosis. *Indicates significant difference between groups on this day. Differences within group among days: a versus b ($P < 0.05$), x versus y ($P < 0.1$). Error bars are SEM.

by JMP 17 software (SAS Institute Inc.). Additionally, numbers of heifers correctly identified as pregnant (true positive [**TP**]), incorrectly identified as pregnant (false positive [**FP**]), correctly rejected as pregnant (true negative [**TN**]), and incorrectly rejected as pregnant (false negative [**FN**]) were calculated in the same package to test performance parameters. Sensitivities, specificities, positive predictive value (**PPV**), negative predictive value (**NPV**), and accuracy were also determined for each day as reported by Pugliesi et al. (2014).

Serum P4 concentrations were greater in pregnant than in control heifers (6.1 ± 0.32 vs. 4.1 ± 0.36 ng/mL, $P = 0.0024$). Even though there was no effect of day, the interaction between group and day tended to be significant ($P = 0.067$): control heifers presented decreased P4 concentrations on d 18 compared with previous days ($P < 0.05$) and pregnant heifers ($P = 0.0007$, Figure 1A). The cervical relative expression of *PGR* mRNA had no significant effect of group or day, but the interaction between group and day tended to be significant ($P = 0.078$), as pregnant heifers maintained their *PGR* cervical expression, whereas control heifers tended to increase *PGR* mRNA expression on d 18 ($P = 0.089$). On d 18 control heifers had greater *PGR* cervical expression than pregnant heifers ($P = 0.031$, Figure 1B). The relative expression of *ISG15* mRNA was greater in pregnant than control heifers (1.89 ± 0.49 vs. 0.13 ± 0.04, respectively, $P < 0.0001$), being different in each day. Pregnant heifers presented 12.0-, 21.5-, and 12.1-fold greater *ISG15* expression on d 14, 16, and 18, respectively, relative to control heifers. The interaction between group and day tended to be significant ($P = 0.059$), as pregnant heifers presented greater *ISG15* mRNA expression on d 16 than on d 14 ($P = 0.0075$), whereas the expression on d 18 was not different from the other days (Figure 2A). No differences among days were detected in control heifers. The ROC curve analysis (Table 1) indicated that *ISG15* relative expression was a significant predictor of pregnancy on d 14 (area under the curve [**AUC**] = 0.86, $P = 0.0027$), d 16 (AUC = 1.0, $P < 0.0001$), and d 18 (AUC = 0.93, $P = 0.0009$), as well as *PGR* relative expression on d 18 (AUC = 0.73, $P = 0.0417$).

The characterization of the physiological scenario through P4 concentrations and *PGR* expression followed the expected results widely described by the literature. Control heifers had decreased P4 concentrations on d 18 when compared with previous days, and on this day it was lower than pregnant heifers, showing the initiation of luteolysis (Lukaszewska and Hansel, 1980). Although we did not find other reports of mRNA *PGR* expression in cervical cells during early pregnancy, in control heifers it tended to increase at the end of the estrous cycle, consistent with the cyclic changes and the cease of the known P4 downregulation on its own receptors as described for the uterus (Meyer et al., 1988; Kimmins and Maclaren, 2001; Meikle et al., 2001). Nevertheless, there was a marked individual variation in cervical *PGR* expression in control heifers on d 18 (Figure 1C), leading to low specificity and lower accuracy of this indicator as a pregnancy diagnostic tool on d 18 (Table 1). The variation in *PGR* expression in control heifers on d 18 could be associated with individual differences in P4 concentrations and timing of luteolysis (Ginther et al., 1989). To our knowledge, this is the first report showing greater *ISG15* mRNA expression in cervical samples as early as d 14 of pregnancy, in comparison to control sham-inseminated heifers. Moreover, as far as we know, this is the first report of the profile of cervical *ISG15* expression in pregnant heifers across days (repeated measures) around maternal recognition of pregnancy. Most of the studies using the traditional methodology of measurement of *ISG15* expression in blood immune cells reported differences from d 18 to 20 of pregnancy (Gifford et al., 2007; Green et al., 2010; Pugliesi et al., 2014; Melo et al., 2020a), with accuracy of pregnancy diagnosis ranging from 70% to 80% in either lactating dairy cows (Han et al., 2006; Yoshino et al., 2018) or beef cattle (Pugliesi et al., 2014; Melo et al., 2020a). It is suggested that tissues with closer proximity to the embryo may have increased expression of ISG

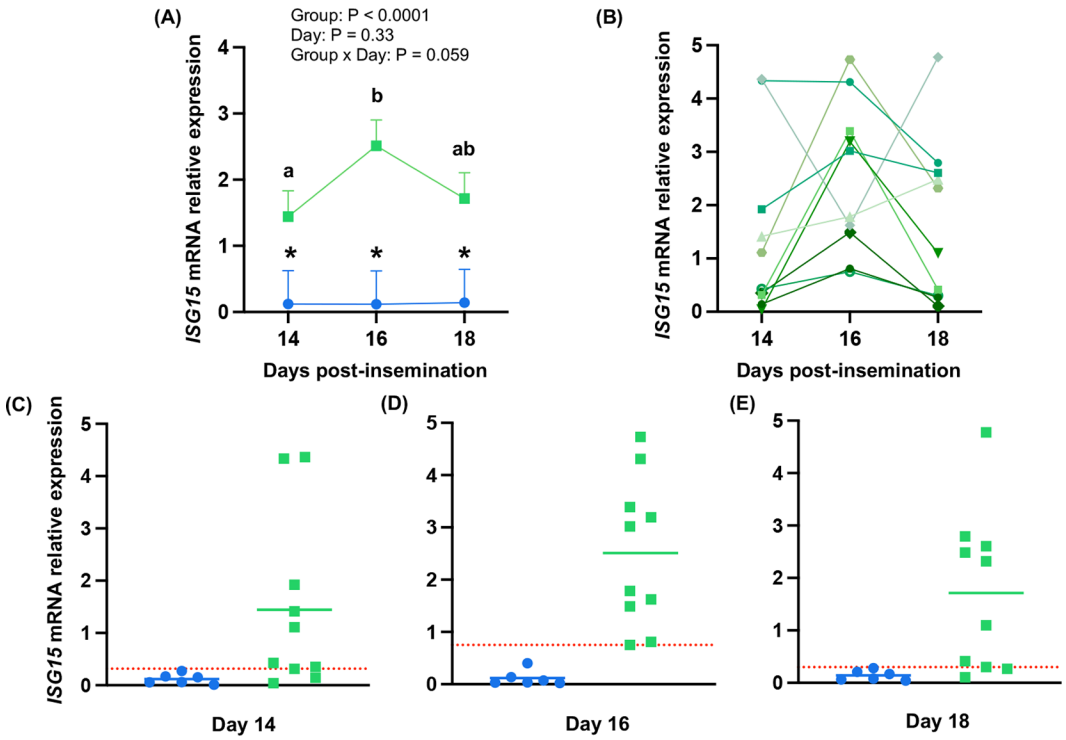

Figure 2. Relative mRNA expression of *ISG15* in control (blue) and pregnant (green) heifers (A) and individual *ISG15* relative mRNA expression of each pregnant heifer (B) on d 14, 16, and 18 postinsemination, and scatter plots with individual distribution of *ISG15* relative mRNA expression in control (blue) and pregnant (green) heifers on d 14 (C), 16 (D), and 18 (E) postinsemination. Dots show the individual values. Means are indicated by the continuous midlines. Red dotted lines indicate the threshold for pregnancy diagnosis. *Indicates significant difference between groups on this day. Differences within group among days: a versus b ($P < 0.05$). Error bars are SEM.

earlier in pregnancy, in concordance with Sponchiado et al. (2017). Other studies found pregnancy-associated greater expression of *ISG15* mRNA in cervix of lactating Holstein cows from d 17 to 18 (Kunii et al., 2018; Domingues et al., 2024) or Holstein heifers and lactating cows on d 20 postinsemination (Ferraz et al., 2021). These studies differed in that pregnant cows were compared with

Table 1. Number of animals, true positive (TP), true negative (TN), false positive (FP), and false negative (FN) diagnoses, and sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and area under the curve (AUC) for determining pregnancy status on d 14, 16, and 18 post-AI by *ISG15* and on d 18 by *PGR* mRNA relative expression

	ISG15			*PGR*
Item	d 14	d 16	d 18	d 18
No. of animals	16	16	16	16
TP	8	10	8	10
TN	6	6	6	3
FP	0	0	0	3
FN	2	0	2	0
Sensitivity[1] (%)	80.0	100	80.0	100
Specificity[2] (%)	100	100	100	50.0
PPV[3] (%)	100	100	100	76.9
NPV[4] (%)	75.0	100	75.0	100
Accuracy[5] (%)	87.5	100	87.5	81.3
AUC (%)	86.0	100	93.3	73.3
P-value	0.0027	<0.0001	0.0009	0.0417

[1]Sensitivity (probability that a test result will be positive when the cow is pregnant) = TP/(TP + FN).

[2]Specificity (probability that a test result will be negative when the cow is not pregnant) = TN/(FP + TN).

[3]PPV (probability that the cow is pregnant when the test is positive) = TP/(TP + FP).

[4]NPV (probability that the cow is not pregnant when the test is negative) = TN/(FN + TN).

[5]Accuracy = (TP + TN)/n (Pugliesi et al., 2014).

cows also inseminated with regular semen but were not pregnant at ultrasound around d 30. The fold-change found in the present study is lower than other reported previously, between 50- and 80-fold (Kunii et al., 2018; Domingues et al., 2024), but greater than the reported by Ferraz et al. (2021) of 3.4-fold. Differences could be due to the type and day of tissue sampling as well as PCR analysis methodology. The greater fold-change between pregnant versus control heifers in the present study was on d 16 of pregnancy, whereas on d 14 and 18 the lower fold-change was also accompanied by a greater individual variability of *ISG15* expression in the pregnant group (Figure 2C–E, Table 1). On d 16 there was no FP or FN (100% of accuracy), whereas on d 14 and 18 postinsemination the accuracy of the diagnosis was acceptable (87.5% in both days). The AUC reached in the present study were greater than those reported by Ferraz et al. (2021) for the same tissue, which could be explained by the day of sampling and the use of control sham-inseminated animals versus nonpregnant animals that could have undergone early embryo losses around or after the sampling time and before the ultrasonography diagnosis. The good specificity and PPV reached with the present data on all days indicate that this biomarker could be a trustable predictor of the positive pregnancy status; however, FN could exist in animals presenting lower expression, which could represent a risk for iatrogenic pregnancy loss if used as a tool for resynchronization protocols. However, further studies with a larger sample size and the inclusion of nonpregnant animals after AI are necessary to determine the accuracy of this methodology. Also, comparisons between heifers and cows are needed because differences between parities in ISG expression in blood cells have been reported (Green et al., 2010; Melo et al., 2020a). The increased *ISG15* expression particularly on d 16 coincides with the moment of maternal recognition of pregnancy in cattle (Hansen et al., 2017), which seems to be a critical stage in the communication of embryo–uterine–ovarian axis (Forde and Lonergan, 2017). In this sense, it was demonstrated in cattle that transfer of embryos to recipients up to d 16 relative to estrus led to a normal pregnancy, while none of the d 17 recipients were pregnant by d 42 (Betteridge et al., 1980). This is in concordance with the peak found on d 16 of pregnancy in studies of repeated measurements of mRNA expression of IFNT in bovine conceptuses and plasma of pregnant cows (Farin et al., 1990; Sheikh et al., 2018). Other works have found increased *ISG15* expression in the endometrium from d 15 to 17 in pregnant compared with nonpregnant cows (Austin et al., 2004; Moraes et al., 2020; Adhikari et al., 2022). However, an investigation in the dynamic temporal pattern of the endometrial transcriptome of pregnant heifers found differences on d 16 but not on d 13 or before, when compared with cyclic heifers (Forde et al., 2011), in disagreement with Sponchiado et al. (2017), which could be attributed to the localization of the samples, the transcriptomic technique, or both. Nonetheless, the present results sustained the hypothesis that IFNT induces the expression of ISG in surrounding reproductive tissues as early as d 14 of pregnancy, and further research could help to elucidate if it can vary depending on the type of cells collected.

In conclusion, cervical cells express greater *ISG15* mRNA in pregnant versus control heifers as early as d 14 postinsemination, with the best accuracy on d 16. Our findings indicated that the technique proposed might serve as a reliable pregnancy diagnostic tool for Holstein heifers, although further validation with a larger sample size is needed, including animals suffering embryo losses and different parities.

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Notes

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Nonstandard abbreviations used: AUC = area under the curve; CASA = computer-assisted semen analyzer; FN = false negative; FP = false positive; IFNT = interferon tau; ISG = interferon-stimulated genes; NPV = negative predictive value; P4 = progesterone; PGR = progesterone receptor; PPV = positive predictive value; qPCR = real-time PCR; ROC = receiver operating characteristic; TN = true negative; TP = true positive.