Gene expression of Pregnancy-associated glycoproteins-1 (PAG-1), Interferon-tau (IFNt) and Interferon stimulated genes (ISGs) as diagnostic and prognostic markers of maternal-fetal cellular interaction in buffalo cows

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Abstract

The aim of this study was to determine the presence of Pregnancy-associated glycoprotein -1 (PAG-1) mRNA expression in the maternal circulation of pregnant buffaloes during the early stage of pregnancy. Contemporaneously, the mRNA expression levels of Interferon-tau (IFNt) and some Interferon stimulated genes (ISGs) (*Interferon stimulated gene 15 ubiquitin-like modifier interferon*, ISG15; *Mixoviruses resistance 1* and *2*, MX1 and MX2; *2',5'-oligoadenylate synthase 1* ,OAS1) were evaluated in order to expand our knowledge of the molecular processes involved in the early stages of pregnancy and to identify potential biomarkers of maternal-fetal cellular interaction in buffalo. The study was conducted on 38 synchronized and artificially inseminated buffalo cows (d 0), divided *ex post* into 3 groups: Pregnant (n=17), Non-pregnant (n=15) and Embryo mortality (n=6). Blood samples were collected on d 14, 19, 28 and 40 after artificial insemination (AI) for peripheral blood mononuclear cells (PBMCs) isolation. Expression levels of mRNA of PAG-1, IFNt, ISG15. MX1, MX2 and OAS1 were measured using RT-qPCR. No significant changes were observed in IFNt and PAG gene expressions between groups, while significant differences ($p<0.001$) were found for

ISG15, MX1, MX2, and OAS1. Pairwise comparisons revealed that the differences between groups occurred on days 19 and 28 post-AI. ISG15 proved to have the best diagnostic performance for distinguishing between pregnant animals and animals that experienced embryo mortality with the ROC analysis. According to the results of the univariate analyses, day 19 was identified as the most indicative to discriminate between groups while the most reliable genes for this differentiation were ISG15, MX1 and MX2. MX2 proved to be the best gene for discriminating pregnant buffaloes using the discriminant analysis, while MX1 was the gene that best predicted embryo mortality. Our results showed that among PAG-1, IFNt and ISGs expression as diagnostic and prognostic markers of maternal-fetal cellular interaction in buffalo cows, ISGs proved to be the best peripheral biomarkers for predicting pregnancy and embryonic mortality during the peri-implantation period. These insights into the mechanisms behind maternal-fetal interaction and the development of a method for the early detection of embryo distress may enable us to implement effective strategies to support embryo survival.

1. Introduction

During pregnancy, proteins play crucial roles as hormones, regulatory and signalling molecules from embryo formation, implantation in the uterus and further development to term [1,2]. Over the years, various molecular complexes that promote pregnancy maintenance have been identified. Starting from the 1980s [3,4], in ruminants the molecules called Pregnancy-associated glycoproteins (PAGs), which are involved in immunological mechanisms in the maintenance of pregnancy and fetal well-being, have been identified [5–7]. These molecules are synthesized by the mononucleate and binucleated trophoblastic cells of species belonging to the order Cetartiodactyla [8–13]. The proteolytic activity that some PAGs share with the aspartic proteinase family members, suggests their possible involvement in placentogenesis, feto-maternal unit remodelling and implantation [14–16]. Once produced and secreted, these glycoproteins enter the maternal bloodstream and can be used as biomarkers for early pregnancy diagnosis in ruminant species [13] including buffalo [8,10,17,18]. Phylogenetic analyses have divided PAGs into two groups: the “ancient” PAGs group (PAG-2), which is deemed to play an important role in implantation and placentation and the “modern” PAGs group (PAG-1), whose function is not entirely clear [13,19]. Detailed information on the expression of PAG-1 mRNA is lacking in ruminants [20,21], especially when detected in peripheral blood cells. To date, the few studies on buffalo PAG gene expression during pregnancy have focused on PAG-2 in maternal blood leukocytes [22] and on PAG-1 on gravid uteruses [23].

Interferon-tau (IFNt) is one of the first molecules involved in early maternal recognition of pregnancy in ruminants [24]. It is secreted by the trophectodermal cells from blastocysts, which generally occurs

between day 16 and day 25 of pregnancy [25]. The role of IFNt is to inhibit oxytocin receptor formation in the endometrium in order to prevent the production of prostaglandin F2-alpha (PGF2α) [26]. This mechanism allows the inhibition of luteolysis, the maintenance of progesterone production by the corpus luteum increasing successful embryo implantation [27]. A recent study that provides strong evidence for the existence of embryo-uterine crosstalk during pre-implantation in buffalo [28], showing that the developing embryo produces IFNt and regulates an immune response by inducing an anti-inflammatory action in uterine epithelial cells for acceptance of the semi-allogenic embryo in the uterus to sustain the pregnancy. Once secreted into the uterine cavity, some of the IFNt enters the uterine vein and stimulates the expression of some genes called interferon-stimulated genes (ISGs), including the *interferon stimulated gene 15 ubiquitin-like modifier interferon* (ISG15), *Mixoviruses resistance 1* and *2* (MX1 and MX2) and *2',5'-oligoadenylate synthase 1* (OAS1) in various cells, namely endometrial cells, peripheral blood cells and luteal cells [29,30]. In cattle, several studies have demonstrated that ISG expression increases in peripheral blood leukocytes during early gestation [31–34], while to date few data are available for buffalo [35–38].

The aim of this study was to evaluated mRNA expression levels of PAG-1, IFNt and some ISGs (ISG15; MX1; MX2 and OAS1) in order to identify potential biomarkers of maternal-fetal cellular interactions in buffalo cows, in an earlier period of gestation respect that in which the proteins can be detect in the maternal blood. To this aim, peripheral blood cells as PBMCs represent a less invasive, and alternative to caruncular tissue, compatible with the maintenance of pregnancy. Identifying relevant circulating biomarkers reflecting the embryo well-being could represent a powerful tool for investigators involved in studying factors affecting embryo mortality.

2. Materials and Methods

2.1 Animals and Experimental Design

The study was carried out at the CREA Animal Production and Aquaculture experimental farm, in Monterotondo, Rome, Italy. The experimental procedures were assessed and approved by the CREA Committee of Ethics in Animal Research (Protocol N.0081676-02/11/2020).

In this study 38 Italian Mediterranean buffalo cows undergoing a synchronization and artificial insemination (AI) program were enrolled and grouped *ex post* as Pregnant (n=17), Non-pregnant (n=15) and Embryo mortality (n=6), based on diagnostic criteria described below in Section 2.2.

The buffalo cows were synchronized with a progesterone-releasing intravaginal device (PRID®) associated with PMSG, PGF2α and GnRH as reported by Barbato et al. [22]. The cows were artificially inseminated using frozen-thawed semen 72 h after PRID® removal. Blood samples were

collected from the jugular vein in 10 mL EDTA tubes on days 14, 19, 28 and 40 post AI, and immediately processed for PBMCs isolation as described below in Section 2.4.

2.2 Pregnancy Diagnosis

Pregnancy was diagnosed using both conventional transrectal ultrasonography on day 30 post AI and PAGs determination.

By transrectal ultrasonography, the buffaloes were considered pregnant if an embryonic vesicle and embryo proper with beating heart were detected, while in the absence of these conditions buffaloes were considered as non-pregnant. When a vesicle without an embryo proper and/or an embryonic heart beat were not visible [39] an embryo mortality was supposed to be occurred and validated with the PAGs results.

PAGs determination was adjunct to conventional ultrasonography to diagnose pregnant and not pregnant animals and monitor the pregnant animals throughout the sampling period to highlight embryo mortality. Blood sample were collected from the jugular vein in 10 mL EDTA tubes on days 0, 14, 19, 28 and 40 post AI, and the plasma was separated by centrifugation at 2700 x g for 10 min and then stored at -20° C until assayed. Based on PAGs plasma levels (cut-off value: 1 ng/mL) [39], the buffaloes were considered non-pregnant when concentrations remained close to zero throughout sampling and pregnant when the concentrations were >1 ng/mL at d 28 and d 40 post-AI. The animals in which PAGs concentration was found close to the cut-off (0.8-1 ng/mL) between 14 and 28, and then dropped below 0.2 ng/mL on day 40, embryo mortality was deemed to have occurred.

PAGs concentration was used to differentiate *ex post* the groups.

2.3 PAGs Radioimmunoassay

RIA-860 previously described by Barbato et al. [10] was used to determine PAGs concentration. Pure boPAG$_{67kDa}$ preparation was used as the standard and tracer. Iodination (Na-I^{125}, Amersham Pharmacia Biotech, Uppsala, Sweden) was carried out according to the Chloramine-T method previously described by Greenwood et al. [40]. The samples were assayed in a preincubated system in which the standard curve ranged from 0.2 to 25 ng/mL. The minimum detection limit (MDL), calculated as the mean concentration minus twice the standard deviation (mean-2 SD) of 20 duplicates of the zero (B0) standard [41], was 0.4 ng/mL. The intra- and inter-assay coefficients were 2.5% and 7.2%, respectively.

2.4 PBMCs isolation

The purification of the PBMCs was obtained following a density gradient using Lymphoprep™ (1.077 g/mL; Axis-Shield). The blood samples (10 mL) were collected from the external jugular vein in vacutainer tubes containing anticoagulant EDTA. The protocol used for isolating cells was described

by Barbato et al. [22]. In short, 8 mL of whole blood was diluted 1:1 with Hank's Buffered Salt Solution (HBSS) (without Ca^{2+} and Mg^{2+}) into 50 mL Falcon® tubes and then carefully layered over 8 mL of Lymphoprep™ at room temperature. The tubes were centrifuged at 800 x g for 30 min at 20° C. The phase containing erythrocytes settled at the bottom of the tube. The phase containing PBMCs settled on the plasma:Lymphoprep™ interface. The upper plasma layer was removed and discarded without disturbing the plasma:Lymphoprep™ interface. The PBMCs layer was collected at the plasma:Lymphoprep™ interface without disturbing the erythrocyte/polymorphonuclear leukocytes pellet. Afterward, the PBMCs were washed twice with HBSS (without Ca^{2+} and Mg^{2+}). Total cell counts and cell viability were determined by LUNA-II™ automated cell counter (Logos Biosystems, Anyang, South Korea). The purity of the isolated PBMCs fraction was evaluated with a CytoFlex flow cytometer (Beckman Coulter) based on the forward scatter channel (FSC) vs the side scatter channel (SSC). PBMCs purity was on average 98%. 5×10^6 cells were lysed in TRIzol™ Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and immediately stored at -80 °C until further investigation analysis.

2.5 RNA extraction

Total RNA was extracted from the PBMCs lysed in 1.0 mL TRIzol™ Reagent according to the manufacturer's protocol. Lastly, the RNA concentration and quality were determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) with the measurement of absorbance and ratio at 260 nm and 280 nm wavelength. In order to ensure the RNA purity and that it is free from DNA contamination, a further treatment was performed with DNase I, RNase-free (1 U/µL) (Invitrogen, Life Technologies, Carlsbad, CA, USA). The samples were then stored at −80° C until the next analysis.

2.6 Reverse-transcription and RT-qPCR

Total RNA (1 µg) was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions. The reverse transcription was performed in the following sequence: step 1, incubation at 25° C for 10 min; step 2 incubation at 37° C for 2 h; and step 3 incubation at 85° C for 5 min.

The cDNA obtained from each sample was used as a template for RT-qPCR. In order to optimized the 25 µl reaction volume in MicroAmp optical 96-well plates, as previously described by Riva et al. [42], each plate contained duplicates of each sample, 2X Power Sybr Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and primers at 500 nM each (0.5 µl of 10 µM solution). The species-specific primer pairs were designed by Primer Express Software (Applied Biosystems, Foster City, CA, USA) using the buffalo sequences taken from NCBI database as target, and the

primer pairs specificity checked using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were purchased from IDT™ (Integrated DNA Technologies, Coralville, Iowa, USA).

The primer sequences are listed in **Table 1**. *BuPAG 18* isophorm was selected for PAG-1 group, because among those present in the database is the earliest expressed. Relative quantification of PAG-1, IFNt and ISGs transcript was carried out following the MIQE guidelines [43].

A duplicate no-template control (NTC) was also included on each plate. RT-qPCR was carried out using the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) at the following thermal cycle conditions: 10 min at 95° C followed by 40 cycles of 15 s at 95° C and 1 min at 60° C. Quantification was determined after applying an algorithm to the data analysed by the software of the 7000 Sequence Detection System (Applied Biosystem, Foster City, CA, USA).

Sample amplification specificity was verified by dissociation curves and agarose gel electrophoresis. Prior to sequencing, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each PCR-fragment was sequenced and its sequence aligned to the corresponding reference sequence before conducting the qPCR-analyses to assess the amplification of the correct gene.

qPCR amplification efficiency was determined using the slope of the standard curve: Efficiency = $10^{-1/\text{slope}} - 1$. Only PCR reactions with PCR efficiencies > 95% were included in the subsequent analyses. In detail, the amplification efficiency was: for the IFNt E= 112.7%; ISG15 E= 104.62%, for MX1 E=101.91%, for MX2 E=95.72%, for OAS1 E= 103.27%, for PAG 18 E=144.12%, and finally for ACTB the amplification efficiency was 98.80%.

The expression of buffalo target genes was normalized to beta-actin (ACTB) reference gene levels (mean) of the same sample and run. The $2^{-\Delta Ct}$ method was used to calculate the relative expression levels of the target genes [44]. The obtained value was multiplied by 10.000 in order to obtain the test Arbitrary Units (AU).

2.7 Statistical Analysis

Diagnostic graphics were used to check assumptions and outliers. Gene expressions and PAG plasma concentrations were analysed by the Generalized Linear Model (GLM) using gamma as probability distributions and log as link function as these variables were positively skewed with a long tail on the right [45]. Animals were included in the models as subjects while Time was included as repeated effects. The models evaluated the main effects of Time (4 levels: 14, 19, 28 and 40 days post-AI), Group (3 levels: Pregnant, Non-pregnant, and Embryo mortality), and their interaction. The last significant difference was used for carrying out multiple comparisons.

Receiver Operating Characteristic (ROC) analyses and the Youden index were used to evaluate the diagnostic performance of each gene and to determine their optimal cut-off value to distinguishing between pregnant and non-pregnant cows and pregnant cows and cows experiencing embryonic mortality [22,46].

Based on the results of the univariate analysis, a genes panel that best discriminates among groups was selected. The discriminant analysis (DA) was then performed to identify the linear combinations of genes (discriminant functions (Dfs)) that best discriminate between Pregnant, Non-pregnant, and Embryo mortality groups. The DA also classified the genes according to their importance in this discrimination. The relative contribution of each gene in classifying the buffalo cows was evaluated using the structure coefficients (correlations between each independent variable and the Df). The centroids indicated the mean discriminant scores of Df for pregnant, non-pregnant, and buffaloes that experienced embryo mortality, which were used to establish the cut-point for classifying samples during cross-validation. The performance of the DA was evaluated by classification-related statistics [47,48].

The statistical analyses were performed with SPSS 25.0 software (SPSS Inc. Chicago, IL, USA). A p value ≤ 0.05 was considered to be statistically significant and $p<0.1$ indicated a trend.

3. Results

3.1 Univariate analyses

No significant changes were found in IFNt and PAG gene expressions (p value for all the effects >0.1; **Figure 1**) while all the evaluated effects (i.e., Time, Group, and their interaction) were significant at 0.1% significance level for ISG15, MX1, MX2, and OAS1.

Pairwise comparisons pointed out that the differences between groups occurred on days 19 and 28 post-AI. In particular, the ISG15, MX1, MX2, and OAS1 expression levels of pregnant buffalo cows were found to be highest on days 19 and 28 ($p<0.001$). The buffalo cows that experienced embryo mortality tended to have higher values of ISG15, MX1 and MX2 than non-pregnant animals on day 19. Their expressions decreased on day 28 ($p<0.1$), and other differences between the embryo mortality and non-pregnant groups were not observed (**Figure 1**).

3.2 ROC analyses

ROC analyses were performed using the data of day 19 post-AI when previous analyses showed the greatest between-groups differences. **Table 2** and **Figure 2** show the area under the ROC curves and the cut-off value of the examined genes. ISG15, MX1, and MX2 were perfectly capable of distinguishing pregnant from non-pregnant buffalo cows (for each gene, AUC=1.000) and, showed

100% sensitivity and specificity at their optimal cut-off value. OAS1 also proved to have excellent diagnostic performances (AUC=0.996) while the diagnostic performance of IFNt and PAG performed poor in all classifications.

ISG15 proved to have the best diagnostic performance for distinguishing pregnant cows from cows that experienced embryonic mortality (AUC=0.980, $p<0.01$). The optimal cut-off value showed a sensitivity and a specificity of 88.2% and 100%, respectively.

The performance of genes to distinguish Non-pregnant from Embryo mortality groups was generally lower. The highest accuracy was found for MX2 (AUC=0.789, $p<0.05$) and the optimal cut-off value showed 66.7% of sensitivity and 93.3% of specificity.

3.3 Discriminant analysis

Based on the results of univariate analyses, day 19 proved to be the most indicative for differentiating the groups, while ISG15, MX1 and MX2 were identified as the best genes for this differentiation. Therefore, the DA was performed using the data collected on day 19 and including the above-mentioned genes as independent variables.

Centroids (**Table 3**) and the plot of Df scores (**Figure 3**) indicated that Df1 was able to discriminate the pregnant cows from the others (i.e., non-pregnant cows and those experiencing embryonic mortality). The results of the multivariate analysis showed that MX2 was the gene that best discriminated pregnant cows (followed by MX1 and ISG15). The second Df separated the Embryo mortality group (with a positive coefficient) from the others (i.e., Pregnant and Non-pregnant groups showing negative coefficients). MX1 was the gene that best discriminated embryo mortality (followed by ISG15 and MX2). Overall, the DA correctly classified 76.3% of the buffalo cows.

4. Discussion

This study investigated the mRNA expression of two molecules that play key roles in maternal-fetal recognition namely PAG-1 and IFNt, as well as the mRNA expression of several IFN-stimulated genes (ISG15; MX1; MX2 and OAS1) on blood circulating cells in order to identify potential biomarkers reflecting embryo well-being during early pregnancy in buffalo.

To our knowledge no previous research has investigated PAG-1 mRNA expression in circulating PBMCs of buffalo cows.

In this study, the PAG-1 expression levels (*BuPAG 18* isoform) in PBMCs did not show statistical differences over all time points and groups analyzed. This lack of difference may be due to the fact that PAGs belong to a very large and complex family of genes [13]. The PAGs family exhibits a spatially and temporally distinct expression patterns during pregnancy: PAG-2 group expression

occurs throughout the outer epithelial layer of the placenta (trophectoderm), while PAG-1 group expression is restricted to the binucleate cells [49,50]. Consequently, some PAGs are expressed in certain stages of pregnancy and not in others. In bovine cotyledon tissues, Wiedemann et al. [21] observed high expression levels in middle and late pregnancy for the PAG-1 group and in early pregnancy for the PAG-2 group. Therefore, PAG-1 group is supposed to play an essential role in the maintenance of pregnancy, while the PAG 2 group is supposed to play an important role in placentation and the maintenance of early pregnancy [20,51,52]. However, these findings derived from studies conducted on PAG mRNA expression in placental tissue. Research carried out on PAG-2 mRNA expression in circulating blood cells reported in our previous study [22] highlights early PAG-2 expression on day 14 post AI in pregnant buffalo. This result is in line with those found by other authors on placental tissue: days 17-19 in bovine [53], day 18 in caprine [49] and day 13 in sheep [50], which coincide with the beginning of implantation.

These findings confirm that PAG-1 expression occurs later in pregnancy in buffalo as in other animal species, which is in line with the results obtained by Lotfan et al. [23], who found high levels of *BuPAG 18* isoform expression starting from day 45 of pregnancy in buffalo. Therefore, our results could suggest that PAG-1 is required for the maintenance of pregnancy rather than for pregnancy establishment.

As previously reported for PAG-1 expression, the results obtained for IFNt expression showed no significant differences between the groups and time points considered. In fact, according to our results it is difficult to find INFt gene expression in blood cells. Actually, despite the fact that IFNt transits from the uterus into the systemic circulation to exert its effects on maternal physiology, it has been noted that IFNt expression in blood is not easily detectable [54,55]. Therefore, it is preferable to measure the total protein in blood.

However, in our recent study [38] significant differences in INFt expression were observed in PBMCs between pregnant and non-pregnant buffaloes on days 28 and 40 after AI. In our opinion, these dissimilarities may be due to differences between the methodology applied in this study and the methodology used in our previous study such as different primer-sequence (Fw: CCATTCTGACCGTGAAGAAGTA- Rev: TCATCTCCACTCTGATGATTTCC, (RefSeq.: *AY535404.1*) [38] vs Fw: CATCTTCCCCATGGCCTTC - Rev: CAACCAAGAGATTCTGCCGG, (RefSeq. *XM_025281435.1*) respectively) and different fluorescence detection methods in qPCR (Probe system *vs* Sybr Green, respectively).

Since it is difficult to evaluate the direct expression of IFNt in blood, the response of circulatory leucocytes to IFNt through the Interferon-Stimulated Genes (ISGs) was used as an alternative method for IFNt expression evaluation. In fact, Matsuyama et al. [33] detected a significant increase of ISGs

mRNA expressions in PBMCs, especially ISG15 and MX2, following exogenous IFNt administration on days 16-17 after standing oestrus in synchronized and artificially inseminated cows. This result is in line with the findings of Oliveira et al. [56] and Bazer et al. [57] who observed that ISGs expression during early pregnancy in sheep is induced by the endocrine release of IFNt from the uterine vein. These findings suggest that the quantity of IFNt produced by conceptus could be monitored indirectly. In our study, all of the ISGs analyzed were more strongly expressed in the Pregnant group than in the Non –pregnant and Embryo mortality groups, and were highly expressed on d 19 and d 28 post AI. These results are in line with the observations by Thakur et al. [36] and Mishra and Sarkar [58], who reported that the gene expression profiles of OAS1, MX2 and ISG15 in buffalo PBMCs increased between days 14 and 20 of pregnancy and declined thereafter. Our findings are also in agreement with the results reported by Nag et al. [37], which showed a significant increase in the expression of ISG15 in buffalo PBMCs on days 18 and 20 and of MX1 on days 14-20 of pregnancy. Recently, Huidrom et al. [28] investigating the effect *in vitro* of pre-implantation embryo on the ISGs expression in buffalo, also found that ISG15, MX2 and OAS1 were upregulated in PBMCs when treated with conditioned medium derived from embryos culture. Still with regard to buffalo, Jain et al. [35] reported that during early pregnancy ISG15 was detected in different reproductive tissues.

A substantial body of literature on other ruminants shows also that the gene expression of MX-1 and OAS-1 genes in peripheral blood is higher in pregnant than in non-pregnant animals during early pregnancy [29,34,59,60]. Studies conducted on cattle in early pregnancy have shown that ISGs are up-regulated in leukocytes and the mean blood levels of ISG15 mRNA of pregnant cows are higher from days 15 to 32 of gestation compared to non-pregnant cows [32]. In addition to ISG15, the same results were also observed for MX1, MX2 and OAS1 on day 18 of pregnancy [31,61,62]. In sheep, 15 to 30 days after insemination, higher levels of MX1 mRNA in PBMCs were found in pregnant than in non-pregnant ewes [63].

To our knowledge, no data have been reported in literature regarding a cut-off value for predicting pregnancy or embryonic mortality for the examined genes in buffalo.

Our results showed that all ISGs were perfectly capable of distinguishing pregnant from non-pregnant animals on day 19 post AI, while IFNt and PAG genes performed poorly. The ROC analysis showed that the MX genes are the most reliable markers for distinguishing pregnant animals and those that experienced embryonic mortality, which was also determined using the discriminant analysis. ISG15 proved to have the best diagnostic performance for discriminating pregnant cows and animals that experienced embryo mortality using the ROC analysis.

By measuring ISGs expression in bovine blood to diagnose pregnancy within 18-20 days post-AI, Green et al. [32] reported an optimal cut-off value for d 18 of pregnancy using OAS1 for heifers while there was a lesser response for cows that reduced the sensitivity of the ISG test on that day.

Yoshino et al. [64], suggested that the ISG15 and MX2 values in blood were reliable indicators of pregnancy between 20 and 22 days post-AI in cows, with 80% accuracy.

In our study, buffalo cows that experienced embryo mortality tended to have higher ISG15, MX1 and MX2 values than non-pregnant cows on day 19. The genes that showed the best diagnostic performance (i.e., ISG15, MX1, and MX2) were then selected for the Discriminant analysis (DA). The DA was conducted with the gene expression recorded on day 19 post AI, when between-group differences were greatest. Discriminant analysis is a multivariate technique that identifies the combinations of variables that best discriminate between groups. We hypothesized that a combination of genes could discriminate between pregnant and non-pregnant buffalo cows and cows that experienced embryo mortality. This analysis could also classify the genes according to their importance in this discrimination. Our findings indicated that MX2 is the gene that best distinguishes the pregnant buffalos from the other group (i.e., non-pregnant ones and those experiencing embryonic mortality). This finding is in line with the observations made by Buragohain et al. [65] who showed an increase of MX2 gene expression in the peripheral blood of pregnant buffalo cows on days 14 and 28, with a ten-fold and fourteen-fold increase in plasma protein levels, respectively. This trend of MX2 expression was also observed in our previous study [38] in which mRNA ISG expression was either evaluated in PBMCs or PMNs.

In cattle, Miagawa et al. [66] and Pugliesi et al. [67] reported MX2 expressed at higher levels in PBMC samples starting from day 15 which peaked on day 20 of pregnancy. The high gene expression of MX2 in pregnant animals could due to the fact that blastocyst elongation coincided with these days, which corresponds to the period when the conceptus synthesizes large amounts of IFNt [24].

In our study, the gene that could best predict embryo mortality according to the multivariate analysis proved to be MX1 on day 19 post AI. In cattle, Sheikh et al. [55] showed that MX1, ISG15 and OAS1 showed higher levels of expression in pregnant cows between 14 and 21 days of gestation than in cows with fertilization failure or embryonic mortality. Studies conducted by Kose et al. [68] in sheep showed that MX1 and ISG15 expression could be used as markers for embryo mortality after observing that the expression of these genes was up-regulated on day 15 and remained high until day 23 and only decreased on day 25 in the group that experienced embryonic mortality.

The results of the discriminant analysis showed that MX2 and MX1 were the best genes to discriminate pregnancy or embryonic mortality, respectively, which may be due to the fact that MX gene expression is up-regulated during early pregnancy to block potential viral infections that could

lead to pregnancy loss [67,69] or because of their role in regulating endometrial secretion, uterine remodeling and anti-luteolytic activities [70]. It has been suggested that MX proteins possess GTPase activity which might be involved in the calcium metabolism of endometrial cells during early pregnancy [71] and might trigger maternal neutrophil migration towards the developing embryo [62].

5. Conclusions

Our results indicated that when PAG-1, IFNt and ISGs expression were used as diagnostic and prognostic markers of maternal-fetal cellular interaction in buffalo cows, ISGs proved to be more reliable peripheral biomarker for the prediction of pregnancy and embryonic mortality in buffalo during the peri-implantation period. These insights into the mechanisms underlying maternal-fetal interaction and the identification of a strategy for the early detection of fetal distress could prove useful for embryo survival.

Although a larger sample size of animals is required to provide more accurate values, these preliminary results may help us to identify a biomarker capable of correctly predicting reproductive failure in early pregnancy, which could facilitate the implementation of reparative mechanisms with the aim of improving fetal well-being and reproductive performance in buffalo cows.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CRediT author statement

A.B. Casano, **V.L. Barile** and **O. Barbato**: Conceptualization, Methodology, Writing-Original Draft; **L. Menchetti**: Formal analysis, Visualization; **M. Trabalza-Marinucci**: Writing - Review & Editing; **F. Riva** and **G. De Matteis**: Investigation and Writing - Review & Editing; **G. Brecchia**: Writing - Review & Editing; **E. Rossi**, **F. Signorelli** and **A. Inglesi**: Investigation.

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References

[1] Khatib H, Huang W, Mikheil D, Schutzkus V, Monson RL. Effects of signal transducer and activator of transcription (STAT) genes STAT1 and STAT3 genotypic combinations on fertilization and embryonic survival rates in Holstein cattle. J Dairy Sci 2009;92:6186–91. https://doi.org/10.3168/jds.2009-2439.

[2] Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PL, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 2016;86:239–53.

[3] Butler JE, Hamilton WC, Sasser RG, Ruder CA, Hass GM, Williams RJ. Detection and partial characterization of two bovine pregnancy-specific proteins. Biol Reprod 1982;26:925–33. https://doi.org/10.1095/biolreprod26.5.925.

[4] Zoli A.P., Beckers JF, Wouters-Ballman P., Closset J., Falmagne P., Ectors F. Purification and Characterization of a Bovine Pregnancy-Associated Glycoprotein. Biol Reprod 1991;45:1–10. https://doi.org/10.1095/biolreprod45.1.1.

[5] Roberts RM, Xie S, Mathialagan N. Maternal recognition of pregnancy. Biol Reprod 1996;54:294–302. https://doi.org/10.1095/biolreprod54.2.294.

[6] Austin KJ, King CP, Vierk JE, Sasser RG, Hansen TR. Pregnancy-Specific Protein B Induces Release of an Alpha Chemokine in Bovine Endometrium. Endocrinology 1999;140:542–5. https://doi.org/10.1210/endo.140.1.6608.

[7] Dosogne H, Burvenich C, Freeman AE, Kehrli Jr ME, Detilleux JC, Sulon J, et al. Pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early post-partum dairy cows. Vet Immunol Immunopathol 1999;67:47–54. https://doi.org/10.1016/S0165-2427(98)00215-3.

[8] Barbato O, Sousa NM, Klisch K, Clerget E, Debenedetti A, Barile VL, et al. Isolation of new pregnancy-associated glycoproteins from water buffalo (Bubalus bubalis) placenta by Vicia villosa affinity chromatography. Res Vet Sci 2008;85:457–66. https://doi.org/10.1016/j.rvsc.2008.01.004.

[9] Barbato O, Sousa NM, Debenedetti A, Canali C, Todini L, Beckers JF. Validation of a new pregnancy-associated glycoprotein radioimmunoassay method for the detection of early pregnancy in ewes. Theriogenology 2009;72:993–1000. https://doi.org/10.1016/j.theriogenology.2009.06.020.

[10] Barbato O, Melo de Sousa N, Barile VL, Canali C, Beckers JF. Purification of pregnancy-associated glycoproteins from late-pregnancy Bubalus bubalis placentas and development of a radioimmunoassay for pregnancy diagnosis in water buffalo females. BMC Vet Res 2013;9:89. https://doi.org/10.1186/1746-6148-9-89.

[11] Barbato O, Menchetti L, Sousa NM, Malfatti A, Brecchia G, Canali C, et al. Pregnancy-associated glycoproteins (PAGs) concentrations in water buffaloes (Bubalus bubalis) during gestation and the postpartum period. Theriogenology 2017;97:73–7. https://doi.org/10.1016/j.theriogenology.2017.04.021.

[12] Nguyen VH, Barbato O, Bui XN, Beckers J-F, Sousa NM de. Assessment of pregnancy-associated glycoprotein (PAG) concentrations in swamp buffalo samples from fetal and maternal origins by using interspecies antisera. Anim Sci J 2012;83:683–9. https://doi.org/10.1111/j.1740-0929.2012.01009.x.

[13] Wallace RM, Pohler KG, Smith MF, Green JA. Placental PAGs: gene origins, expression patterns, and use as markers of pregnancy. Reproduction 2015;149:R115-126. https://doi.org/10.1530/REP-14-0485.

[14] Telugu BPVL, Walker AM, Green JA. Characterization of the bovine pregnancy-associated glycoprotein gene family--analysis of gene sequences, regulatory regions within the promoter and expression of selected genes. BMC Genomics 2009;10:185. https://doi.org/10.1186/1471-2164-10-185.

[15] Telugu BPVL, Palmier MO, Doren SRV, Green JA. An examination of the proteolytic activity for bovine pregnancy-associated glycoproteins 2 and 12. Biol Chem 2010;391:259–70. https://doi.org/10.1515/bc.2010.016.

[16] Jerome A, Singh SK, Agarwal SK, Saini M, Raut A. Characterization and In Silico Analysis of Pregnancy-Associated Glycoprotein-1 Gene of Buffalo (Bubalus bubalis). Genet Res Int 2011;2011:436138. https://doi.org/10.4061/2011/436138.

[17] Karen A, Darwish S, Ramoun A, Tawfeek K, Van Hanh N, De Sousa N, et al. Accuracy of ultrasonography and pregnancy-associated glycoprotein test for pregnancy diagnosis in buffaloes. Theriogenology 2007;68:1150–5.

[18] Barbato O, Menchetti L, Brecchia G, Barile VL. Using Pregnancy-Associated Glycoproteins (PAGs) to Improve Reproductive Management: From Dairy Cows to Other Dairy Livestock. Animals 2022;12:2033. https://doi.org/10.3390/ani12162033.

[19] Hughes AL, Green JA, Garbayo JM, Roberts RM. Adaptive diversification within a large family of recently duplicated, placentally expressed genes. Proc Natl Acad Sci U S A 2000;97:3319–23. https://doi.org/10.1073/pnas.97.7.3319.

[20] Patel OV, Yamada O, Kizaki K, Takahashi T, Imai K, Hashizume K. Quantitative analysis throughout pregnancy of placentomal and interplacentomal expression of pregnancy-associated glycoproteins-1 and -9 in the cow. Mol Reprod Dev 2004;67:257–63. https://doi.org/10.1002/mrd.20017.

[21] Wiedemann I, Krebs T, Momberg N, Knorr C, Tetens J. mRNA expression profiling in cotyledons reveals significant up-regulation of the two bovine pregnancy-associated glycoprotein genes boPAG-8 and boPAG-11 in early gestation. Vet Med Sci 2018;4:341–50. https://doi.org/10.1002/vms3.123.

[22] Barbato O, Guelfi G, Menchetti L, Brecchia G, Melo de Sousa N, Canali C, et al. Investigation of PAG2 mRNA Expression in Water Buffalo Peripheral Blood Mononuclear Cells and Polymorphonuclear Leukocytes from Maternal Blood at the Peri-Implantation Period. Vet Sci 2019;6:8. https://doi.org/10.3390/vetsci6010008.

[23] Lotfan M, Choudhary S, Yadav ML, Kumar S, Singh S, Bathla S, et al. Primary structures of different isoforms of buffalo pregnancy-associated glycoproteins (BuPAGs) during early pregnancy and elucidation of the 3-dimensional structure of the most abundant isoform BuPAG 7. PLoS ONE 2018;13:e0206143. https://doi.org/10.1371/journal.pone.0206143.

[24] Thatcher WW, Meyer MD, Danet-Desnoyers G. Maternal recognition of pregnancy. J Reprod Fertil Suppl 1995;49:15–28.

488 [25] Saugandhika S, Sharma V, Malik H, Saini S, Bag S, Kumar S, et al. Expression and purification
489 of buffalo interferon-tau and efficacy of recombinant buffalo interferon-tau for in vitro embryo
490 development. Cytokine 2015;75:186–96. https://doi.org/10.1016/j.cyto.2015.03.012.

491 [26] Spencer TE, Bazer FW. Conceptus signals for establishment and maintenance of pregnancy.
492 Reprod Biol Endocrinol 2004;2:49. https://doi.org/10.1186/1477-7827-2-49.

493 [27] Forde N, Lonergan P. Interferon-tau and fertility in ruminants. Reproduction 2017;154:F33–43.
494 https://doi.org/10.1530/REP-17-0432.

495 [28] Huidrom LD, Dhanaji SN, Pandey S, Chandra V, Gutulla TS. Embryo-Uterine Cross-Talk:
496 Exploration of the Immunomodulatory Mechanism in Buffalo. Animals (Basel) 2022;12:3138.
497 https://doi.org/10.3390/ani12223138.

498 [29] Shirasuna K, Matsumoto H, Kobayashi E, Nitta A, Haneda S, Matsui M, et al. Upregulation of
499 interferon-stimulated genes and interleukin-10 in peripheral blood immune cells during early
500 pregnancy in dairy cows. J Reprod Dev 2012;58:84–90. https://doi.org/10.1262/jrd.11-094k.

501 [30] Toji N, Koshi K, Furusawa T, Takahashi T, Ishiguro-Oonuma T, Kizaki K, et al. A cell-based
502 interferon-tau assay with an interferon-stimulated gene 15 promoter. Biomed Res 2018;39:13–
503 20. https://doi.org/10.2220/biomedres.39.13.

504 [31] Gifford CA, Racicot K, Clark DS, Austin KJ, Hansen TR, Lucy MC, et al. Regulation of
505 interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant
506 dairy cows. J Dairy Sci 2007;90:274–80. https://doi.org/10.3168/jds.S0022-0302(07)72628-0.

507 [32] Green JC, Okamura CS, Poock SE, Lucy MC. Measurement of interferon-tau (IFN-tau)
508 stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18-20d after
509 insemination in dairy cattle. Anim Reprod Sci 2010;121:24–33.
510 https://doi.org/10.1016/j.anireprosci.2010.05.010.

511 [33] Matsuyama S, Kojima T, Kato S, Kimura K. Relationship between quantity of IFNT estimated
512 by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic
513 mortality after AI or ET. Reprod Biol Endocrinol 2012;10:21. https://doi.org/10.1186/1477-
514 7827-10-21.

515 [34] Kizaki K, Shichijo-Kizaki A, Furusawa T, Takahashi T, Hosoe M, Hashizume K. Differential
516 neutrophil gene expression in early bovine pregnancy. Reprod Biol Endocrinol 2013;11:6.
517 https://doi.org/10.1186/1477-7827-11-6.

[35] Jain A, Baviskar PS, Kandasamy S, Kumar R, Singh R, Kumar S, et al. Interferon stimulated gene 15 (ISG15): molecular characterization and expression profile in endometrium of buffalo (Bubalus bubalis). Anim Reprod Sci 2012;133:159–68. https://doi.org/10.1016/j.anireprosci.2012.06.023.

[36] Thakur N, Singh G, Paul A, Bharati J, Rajesh G, Gm V, et al. Expression and molecular cloning of interferon stimulated genes in buffalo (Bubalus bubalis). Theriogenology 2017;100:50–8. https://doi.org/10.1016/j.theriogenology.2017.05.027.

[37] Nag BP, Arunmozhi N, Sarath T, Asokan S, Vijayarani K, Monica G, et al. Interferon stimulated gene 15 and Myxovirus resistance 2 genes are upregulated during early pregnancy in buffaloes. Int J Curr Microbiol App Sci 2018;7:1755–62.

[38] Casano AB, Barile VL, Menchetti L, Guelfi G, Brecchia G, Agradi S, et al. Interferon-tau (IFNt) and Interferon-Stimulated Genes (ISGs) Expression in Peripheral Blood Leukocytes and Correlation with Circulating Pregnancy-Associated Glycoproteins (PAGs) during Peri-Implantation and Early Pregnancy in Buffalo Cows. Animals 2022;12:3068. https://doi.org/10.3390/ani12223068.

[39] Barile VL, Menchetti L, Casano AB, Brecchia G, Melo de Sousa N, Zelli R, et al. Approaches to Identify Pregnancy Failure in Buffalo Cows. Animals (Basel) 2021;11:487. https://doi.org/10.3390/ani11020487.

[40] Greenwood FC, Hunter WM, Glover JS. The preparation of 131I-labelled human growth hormone of high specific radioactivity. Biochem J 1963;89:114–23.

[41] Skelley DS, Brown LP, Besch PK. Radioimmunoassay. Clin Chem 1973;19:146–86. https://doi.org/10.1093/clinchem/19.2.146.

[42] Riva F, Rahman MM, Turin L, Ceciliani F, Russo S, Tribbioli G, et al. TIR8 receptor expression in bovine tissues. Vet Immunol Immunopathol 2010;136:65–70. https://doi.org/10.1016/j.vetimm.2010.02.009.

[43] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–22. https://doi.org/10.1373/clinchem.2008.112797.

[44] Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.

[45] Garson GD. Generalized Linear Models/ Generalized Estimating Equations. Asheboro, NC: ed. Statistical Associates Publishers 2013;350.

[46] Greiner M, Pfeiffer D, Smith RD. Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. Prev Vet Med 2000;45:23–41. https://doi.org/10.1016/s0167-5877(00)00115-x.

[47] Garson GD. Discriminant function analysis. Asheboro, NC: Statistical Associates Publishers 2012.

[48] Agradi S, Curone G, Negroni D, Vigo D, Brecchia G, Bronzo V, et al. Determination of Fatty Acids Profile in Original Brown Cows Dairy Products and Relationship with Alpine Pasture Farming System. Animals (Basel) 2020;10:1231. https://doi.org/10.3390/ani10071231.

[49] Garbayo JM, Green JA, Manikkam M, Beckers JF, Kiesling DO, Ealy AD, et al. Caprine pregnancy-associated glycoproteins (PAG): their cloning, expression, and evolutionary relationship to other PAG. Mol Reprod Dev 2000;57:311–22. https://doi.org/10.1002/1098-2795(200012)57:4<311::AID-MRD2>3.0.CO;2-F.

[50] Green JA, Xie S, Quan X, Bao B, Gan X, Mathialagan N, et al. Pregnancy-Associated Bovine and Ovine Glycoproteins Exhibit Spatially and Temporally Distinct Expression Patterns During Pregnancy. Biol Reprod 2000;62:1624–31. https://doi.org/10.1095/biolreprod62.6.1624.

[51] Weems YS, Lammoglia MA, Vera-Avila HR, Randel RD, Sasser RG, Weems CW. Effects of luteinizing hormone (LH), PGE2, 8-Epi-PGE1, 8-Epi-PGF2 alpha, trichosanthin and pregnancy specific protein B (PSPB) on secretion of prostaglandin (PG) E (PGE) or F2 alpha (PGF2 alpha) in vitro by corpora lutea (CL) from nonpregnant and pregnant cows. Prostaglandins Other Lipid Mediat 1998;55:359–76. https://doi.org/10.1016/s0090-6980(98)00030-6.

[52] Touzard E, Reinaud P, Dubois O, Guyader-Joly C, Humblot P, Ponsart C, et al. Specific expression patterns and cell distribution of ancient and modern PAG in bovine placenta during pregnancy. Reproduction 2013;146:347–62. https://doi.org/10.1530/REP-13-0143.

[53] Xie S, Low BG, Nagel RJ, Beckers J-F, Michael Roberts R. A Novel Glycoprotein of the Aspartic Proteinase Gene Family Expressed in Bovine Placental Trophectoderm. Biol Reprod 1994;51:1145–53. https://doi.org/10.1095/biolreprod51.6.1145.

[54] Bott RC, Ashley RL, Henkes LE, Antoniazzi AQ, Bruemmer JE, Niswender GD, et al. Uterine vein infusion of interferon tau (IFNT) extends luteal life span in ewes. Biol Reprod 2010;82:725–35. https://doi.org/10.1095/biolreprod.109.079467.

[55] Sheikh AA, Hooda OK, Kalyan A, Kamboj A, Mohammed S, Alhussien M, et al. Interferon-tau stimulated gene expression: A proxy to predict embryonic mortality in dairy cows. Theriogenology 2018;120:61–7. https://doi.org/10.1016/j.theriogenology.2018.07.028.

[56] Oliveira JF, Henkes LE, Ashley RL, Purcell SH, Smirnova NP, Veeramachaneni DNR, et al. Expression of Interferon (IFN)-Stimulated Genes in Extrauterine Tissues during Early Pregnancy in Sheep Is the Consequence of Endocrine IFN-τ Release from the Uterine Vein. Endocrinology 2008;149:1252–9. https://doi.org/10.1210/en.2007-0863.

[57] Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G. Comparative aspects of implantation. Reproduction 2009;138:195–209. https://doi.org/10.1530/REP-09-0158.

[58] Mishra SR, Sarkar M. Interferon Stimulated Genes (Isgs): Novel Pregnancy Specific Biomarker In Buffaloes (Bubalus bubalis). J Immunol Sci 2018;2.

[59] Johnson GA, Stewart MD, Allison Gray C, Choi Y, Burghardt RC, Yu-Lee L-Y, et al. Effects of the Estrous Cycle, Pregnancy, and Interferon Tau on 2′,5′-Oligoadenylate Synthetase Expression in the Ovine Uterus. Biol Reprod 2001;64:1392–9. https://doi.org/10.1095/biolreprod64.5.1392.

[60] Haq IU, Han Y, Ali T, Wang Y, Gao H, Lin L, et al. Expression of interferon-stimulated gene ISG15 and ubiquitination enzymes is upregulated in peripheral blood monocyte during early pregnancy in dairy cattle. Reprod Biol 2016;16:255–60. https://doi.org/10.1016/j.repbio.2016.10.001.

[61] Melo GD, Pinto LMF, Rocha CC, Motta IG, Silva LA, da Silveira JC, et al. Type I interferon receptors and interferon-τ-stimulated genes in peripheral blood mononuclear cells and polymorphonuclear leucocytes during early pregnancy in beef heifers. Reprod Fertil Dev 2020;32:953–66. https://doi.org/10.1071/RD19430.

[62] Panda BSK, Mohapatra SK, Chaudhary D, Alhussien MN, Kapila R, Dang AK. Proteomics and transcriptomics study reveals the utility of ISGs as novel molecules for early pregnancy diagnosis in dairy cows. J Reprod Immunol 2020;140:103148. https://doi.org/10.1016/j.jri.2020.103148.

[63] Yankey SJ, Hicks BA, Carnahan KG, Assiri AM, Sinor SJ, Kodali K, et al. Expression of the antiviral protein Mx in peripheral blood mononuclear cells of pregnant and bred, non-pregnant ewes. J Endocrinol 2001;170:R7-11. https://doi.org/10.1677/joe.0.170r007.

[64] Yoshino H, Toji N, Sasaki K, Koshi K, Yamagishi N, Takahashi T, et al. A predictive threshold value for the diagnosis of early pregnancy in cows using interferon-stimulated genes in

612 granulocytes. Theriogenology 2018;107:188–93.
613 https://doi.org/10.1016/j.theriogenology.2017.11.014.

614 [65] Buragohain L, Kumar R, Nanda T, Phulia SK, Mohanty AK, Kumar S, et al. Serum MX2 Protein
615 as Candidate Biomarker for Early Pregnancy Diagnosis in Buffalo. Reprod Domest Anim
616 2016;51:453–60. https://doi.org/10.1111/rda.12700.

617 [66] Miagawa BT, França MR, Paiva YN, Oliveira ML, Lopes E, Gonella-Diaza AM, et al. Presence
618 of conceptus modulates expression of interferon-tau stimulated genes in peripheral blood
619 immune cells of pregnant and non-pregnant beef cows. Animal Reproduction; Proceedings of
620 the 27th annual meeting of Brazilian Embryo Technology Society 2013;10:558.

621 [67] Pugliesi G, Miagawa BT, Paiva YN, França MR, Silva LA, Binelli M. Conceptus-induced
622 changes in the gene expression of blood immune cells and the ultrasound-accessed luteal
623 function in beef cattle: how early can we detect pregnancy? Biol Reprod 2014;91:95.
624 https://doi.org/10.1095/biolreprod.114.121525.

625 [68] Kose M, Kaya MS, Aydilek N, Kucukaslan I, Bayril T, Bademkiran S, et al. Expression profile
626 of interferon tau–stimulated genes in ovine peripheral blood leukocytes during embryonic death.
627 Theriogenology 2016;85:1161–6. https://doi.org/10.1016/j.theriogenology.2015.11.032.

628 [69] Gill TJ. Immunity and pregnancy. Crit Rev Immunol 1985;5:201–27.

629 [70] Hicks BA, Etter SJ, Carnahan KG, Joyce MM, Assiri AA, Carling SJ, et al. Expression of the
630 uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J Anim Sci 2003;81:1552–61.
631 https://doi.org/10.2527/2003.8161552x.

632 [71] Ott T, Gerami-Naini B, Kim H, Taylor K, Spencer T, Bazer F. Expression of the antiviral protein
633 Mx in the ovine uterus is regulated by estrogen (E), progesterone (P), and interferon-tau (IFN
634 tau). vol. 60, Soc Study Reproduction - 1603 MONROE ST, MADISON, WI 53711-2021 USA;
635 1999, p. 211–211.

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643 **Tables**

644 **Table 1.** The primer sequences of buffalo assays used in this study.

Name	Sequence (5' → 3')	NCBI RefSeq.	Amplicon
IFNt		**XM_025281435.1**	
Fw	CATCTTCCCCATGGCCTTC		81
Rev	CAACCAAGAGATTCTGCCGG		
ISG15		**NM_001291322.1**	
Fw	AGAACCCACGGCCATGG		121
Rev	GATCTTCTGGGCGATGAACTG		
MX1		**XM_025279556.1**	
Fw	TTGAAAAACGGTATCGTGGCA		131
Rev	TCTTGCTGTCACTGTGCGC		
MX2		**NM_001290849.1**	
Fw	TGCAATCTGCATCAAACAGTCC		121
Rev	GGTCTTGACAAAAAACCAGCTTCT		
OAS1		**XM_025267539.1**	
Fw	ATCGTCCACCCAAGCTGAAG		81
Rev	GCAGGTTATTCTCATGCTCCTTACA		
PAG-1 (BuPAG 18)		**XM_025286464.1**	
Fw	TTTGCACCAGTCCAACCTGTT		81
Rev	AGGTCTTATTGGTAGGCCGGA		
ACTB		**XM_025274489.1**	
Fw	AACCGTGAAAAAATGACCCAGAT		131
Rev	CCGTCACCGGAGTCCATC		

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Table 2. Results of the Receiver Operating Characteristic (ROC) curve analyses. Sensitivity, specificity and area under the curve (AUC) for the optimal cut-off value for the genes under study.

Discrimination	Gene	Area	95% Confidence Interval	Sig.[1]	Optimal Cut-off	Sensitivity (%)	Specificity (%)
Pregnant *vs* Non-pregnant[2]	**IFNt**	0.529	0.313-0.745	0.777	0.20	64.7	53.3
	ISG15	1.000	1.000-1.000	<0.001	253.1	100	100
	MX1	1.000	1.000-1.000	<0.001	995.4	100	100
	MX2	1.000	1.000-1.000	<0.001	568.7	100	100
	OAS1	0.996	0.983-1.000	<0.001	753.6	94.1	100
	PAG	0.476	0.256-0.696	0.827	0.01	80.0	35.7
Pregnant *vs* Embryo mortality[2]	**IFNt**	0.627	0.347 - 0.908	0.363	0.19	64.7	83.3
	ISG15	0.980	0.931 - 1.000	0.001	470.39	88.2	100.0
	MX1	0.882	0.698-1.000	0.006	1241.83	88.2	83.3
	MX2	0.961	0.877 - 1.000	0.001	969.81	76.5	100.0
	OAS1	0.912	0.745 - 1.000	0.003	666.88	100.0	83.3
	PAG	0.452	0.202-0.703	0.741	0.02	57.1	50.0
Non pregnant *vs* Embryo mortality[3]	**IFNt**	0.444	0.199-0.690	0.697	0.12	100.0	26.7
	ISG15	0.700	0.396 - 1.000	0.161	79.71	66.7	93.3
	MX1	0.744	0.509-.980	0.087	830.83	66.7	80
	MX2	0.789	0.566-1.000	0.043	326.34	66.7	93.3
	OAS1	0.689	0.443 - 0.935	0.186	486.59	50.0	80.0
	PAG	0.548	0.297-0.798	0.741	0.01	100.0	35.7

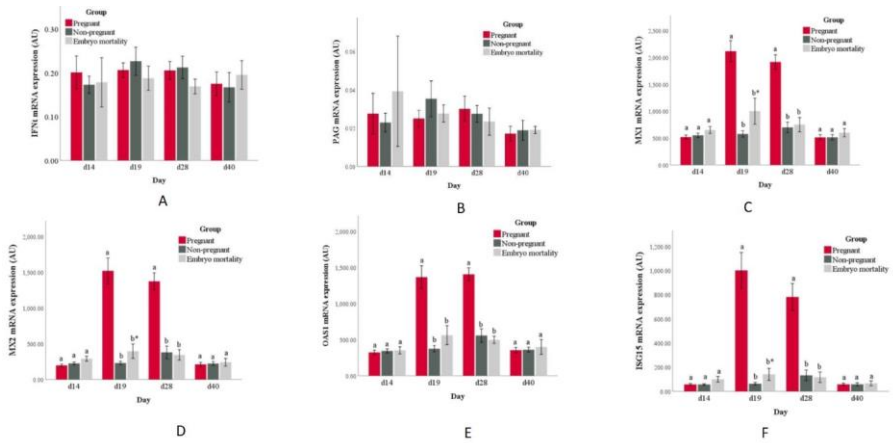
1. Null hypothesis: true area = 0.5

2. The positive actual state is Pregnant.

3 The positive actual state is Embryo mortality.

Table 3. Results of the Discriminant analysis: structure coefficients and centroids of discriminant function 1 (Df1, separating the Pregnant from the Non-pregnant and Embryo mortality groups) and function 2 (Df2, separating Embryo mortality from the Pregnant and Non-pregnant groups).

	Structure Matrix	
Gene	**Df1**	**Df2**
MX2	0.986	-0.077
MX1	0.964	0.256
ISG15	0.893	-0.160
	Functions at Group Centroids	
Group	**Df1**	**Df2**
Pregnant	1.348	-0.050
Non-pregnant	-1.210	-0.290
Embryo mortality	-0.794	0.866



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677 **Figure 1.** IFNt (A), PAG (B), MX1 (C), MX2 (D), OAS1 (E) ISG15 (F) and gene expression in PBMCs of
678 pregnant and non–pregnant buffalo cows and buffalo cows that experienced embryonic mortality. Bars not
679 sharing any superscript within each time are significantly different at $P<0.05$. When letters are not present, no
680 effect was statistically significant.

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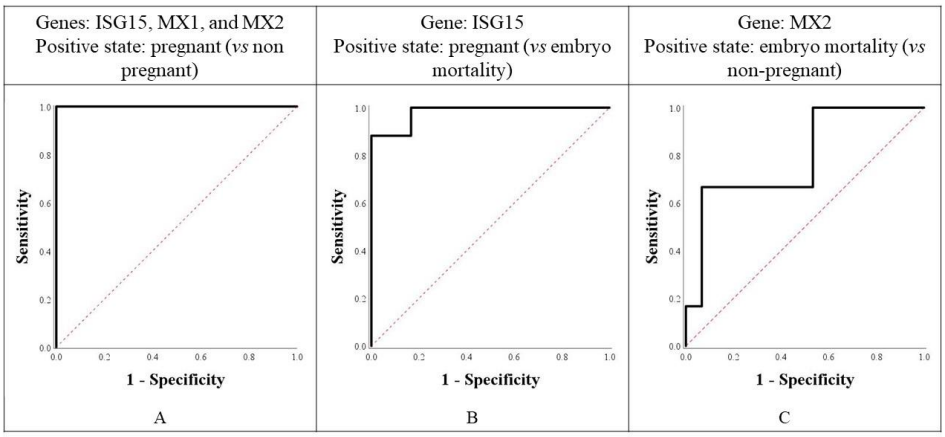

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683 **Figure 2.** Receiver Operating Characteristic (ROC) curves of ISG15, MX1, and MX2 to discriminate between
684 pregnant and non-pregnant buffalo cows (A), ISG15 to discriminate pregnant buffalo cows from buffalo cows
685 that experienced embryonic mortality (B), and MX2 to discriminate non-pregnant buffalo cows from buffalo
686 cows that experienced embryonic mortality (C).

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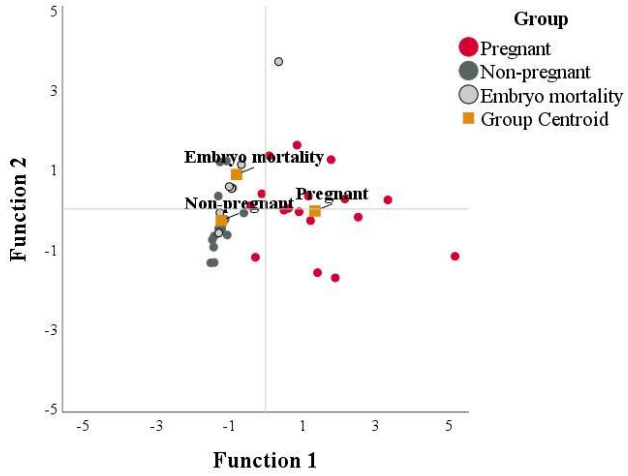

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689 **Figure 3.** Plot of discriminant function scores.

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