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DUPLEX QUANTITATIVE POLYMERASE CHAIN REACTION OF *ISG15* AND *RSAD2* INCREASES ACCURACY OF EARLY PREGNANCY DIAGNOSIS IN DAIRY COWS*

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Abstract

Early diagnosis of pregnancy is important in livestock production, but there is no reliable technology used for pregnancy diagnosis within the first three weeks after insemination. During early pregnancy, the expression of interferon-stimulating genes (ISGs) in peripheral blood leukocytes (PBL) is significantly increased. However, due to different strains, detection sample types, detection methods, threshold value, etc. the specific effectiveness of early pregnancy diagnosis using ISGs is worth further study. The purpose of this study was to test interferon-stimulated protein 15 (*ISG15*), 2'–5'-oligoadenylate synthetase 1 (*OAS1*) and radical S-adenosyl methionine domain containing 2 (*RSAD2*) for early pregnancy diagnosis in dairy cows. The expression of *ISG15*, *OAS1*, and *RSAD2* in PBL of pregnant and non-pregnant heifers on days 0, 14, 18, 21 and 28 after artificial insemination (AI) was detected by fluorescence quantitative polymerase chain reaction (PCR). The sensitivity and specificity of the pregnancy diagnosis was analyzed using expression of these three genes separately or in combination by receiver operating characteristic curve. The combination with the highest accuracy used probe primers and duplex fluorescence quantitative PCR. The single quantitative PCR results showed that expression of *ISG15*, *OAS1* and *RSAD2* on day 18 after AI was significantly higher in pregnant than in non-pregnant cows. When these three genes were used separately, or in combination, for early pregnancy diagnosis, the sensitivity for the *RSAD2* gene was 100%, and the combination of *ISG15* with *RSAD2* was 94.7%. The duplex quantitative PCR showed that, although the sensitivity of *ISG15* alone was 100%, its specificity was only 88.2% (cut-off value 1.402). The sensitivity of *RSAD2* alone was 89.5%, and the specificity was 88.2%; however, when the two genes were used in combination, the sensitivity, specificity and diagnostic cut-off value were consistent with the results of single quantitative PCR. These results indicated that a duplex quantitative PCR assay system for early pregnancy diagnosis in cows using *ISG15* and *RSAD2* was established. Simultaneous detection of expression of *ISG15* and *RSAD2* by duplex quantitative PCR can effectively improve the diagnostic accuracy for dairy cows.

Key words: cow, pregnancy diagnosis, interferon-stimulating gene, duplex quantitative PCR

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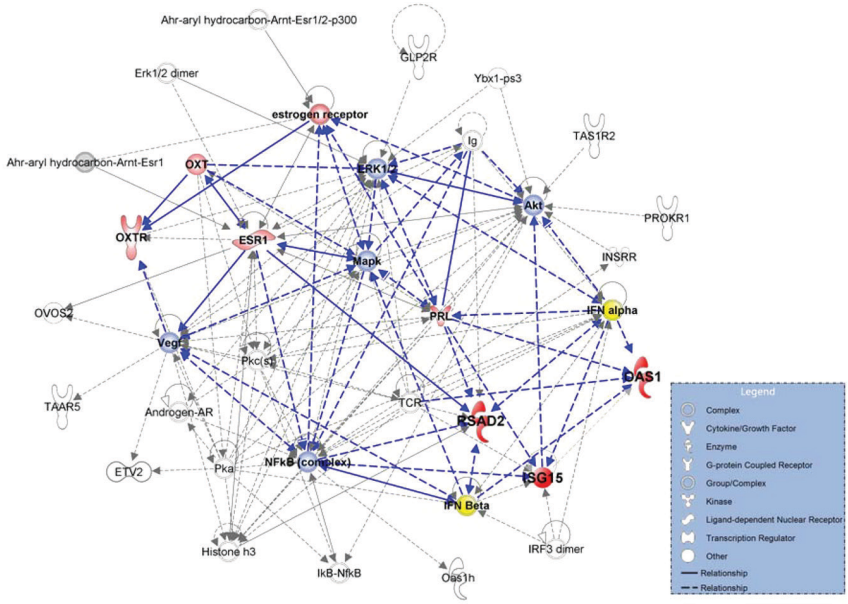
The high-intensity genetic selection of milk production traits eventually leads to adverse effects such as fertilization failure and embryo mortality increase. This is an important reason for the decline in cow fertility (Lucy, 2001; Diskin and Morris, 2008), resulting in economic losses (Lee and Kim, 2007). The ideal breeding target is regular calving, that is, each dairy cow produces one calf per year followed by a 60-day postparturient barren interval. The premise of this goal is the timely implementation of pregnancy diagnosis of the dairy cows after artificial insemination (AI). This would allow identification, and re-insemination, of non-pregnant cows, effectively shortening the calving interval and reducing milk loss. Managing pregnant cows carefully would help, reduce early embryo loss and abortions.

Currently, common pregnancy diagnostic methods include direct and indirect examinations. Direct examination includes rectal and ultrasound examination, which can detect pregnancy on day 35 (Abbitt et al., 1978) and day 27 (Beal et al., 1992; Fricke, 2002) after AI, respectively. Indirect examinations include serum or milk progesterone (P4) and pregnancy-associated glycoproteins (PAGs) detection. Elevated progesterone could also be detected in non-pregnant cows, high progesterone concentrations on days 18–24 after AI are not specific indicators of pregnancy, nevertheless, low progesterone concentrations during this period can accurately predict non-pregnant cows (Balhara et al., 2013). PAGs can be used as biomarkers of pregnancy on day 28 after AI (Friedrich and Holtz, 2010), but the best time for first-time diagnosis of pregnancy using PAGs is day 32 after AI (Ricci et al., 2015). With the continued improvement of PAG assays, pregnancy diagnosis earlier in the 4th week of gestation has increased (Reese et al., 2018). Nonetheless, these methods cannot diagnose pregnancy within one estrus cycle (21 days) after AI. However, 70%–80% of pregnancy failures occur within the first 21 days after fertilization, and early embryonic loss during maternal pregnancy recognition on days 14–19 after AI accounts for most of these failures (Sreenan and Diskin, 1987; Diskin and Morris, 2008; Wiltbank et al., 2016). Accurate diagnosis of pregnancy before day 21 after AI would ensure the timely examination of non-pregnant cows and effectively improve pregnancy rate.

Interferon (IFN)-τ is the most important pregnancy recognition signal in ruminants. It is secreted by the bovine conceptus on days 14–19 after AI. It binds to type I IFN receptor on the endometrium, reduces the expression level of estrogen receptor and oxytocin receptor, inhibits synthesis of prostaglandin $F_{2\alpha}$ and maintains corpus luteum function (Kimura, 2005). Studies have shown that some IFN-τ are secreted into the uterine vein after entering the uterine cavity, resulting in increased expression of interferon-stimulating genes (ISGs) in peripheral blood leucocytes (PBL) and corpus luteum tissue (Bott et al., 2010; Oliveira et al., 2008). Microarray technology has shown that many ISGs are upregulated in PBL during pregnancy recognition (Green et al., 2010 a; Kizaki et al., 2013). Green et al. (2010 a) demonstrated that it is feasible to detect pregnancy of heifers (~1 year old) using ISG expression in PBL. Upregulation of the ISGs during pregnancy provides the possibility of developing new methods for diagnosis of pregnancy, especially within 21 days after AI. However, diagnosis can be challenging, as there is now a large variety of commercial cows, many details including reliable biomarker genes, method of sample collection

and detection, diagnostic criteria, and stability of test results require further study (Toji et al., 2017; Yoshino et al., 2018).



Depicted is the result of the functional analysis of network in which *ISG15*, *OAS1*, and *RSAD2* are involved. Nodes of important molecules are highlighted with colors (yellow/red/green). Nodes are displayed using various shapes that represent the functional class of the gene product as indicated in the legend.

Figure 1. Gene-interaction network analysis of reproductive system development and function

Previous studies showed that expression of interferon-stimulated protein 15 (*ISG15*), 2'–5'-oligoadenylate synthetase 1 (*OAS1*) and radical S-adenosyl methionine domain containing 2 (*RSAD2*) was significantly upregulated on day 16 after pregnancy in endometrial tissue (Forde et al., 2011; Mamo et al., 2012). Our Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) showed that these three genes were closely involved in reproductive system development and function (Figure 1) through interactions with IFN-α and IFN-β. They also indirectly interacted with estrogen and oxytocin receptors, which are important regulators of IFN-τ. Previous studies have confirmed that *ISG15*, *OAS1,* and *RSAD2* are upregulated within 21 days after AI in peripheral blood of pregnant cows, but the extent to which they are upregulated varies (Green et al., 2010 a; Kizaki et al., 2013; Gifford et al., 2007). The latest research shows that *ISG15* mRNA levels are upregulated in peripheral blood of pregnant ewes within 21 days after AI, and if the embryo dies in the early stage of pregnancy, *ISG15* expression is markedly attenuated on the following days (Kose et al., 2016). Although reports have shown that *ISG15* and *OAS1* could be

used as biomarkers for the diagnosis of pregnancy, the results with the two genes are inconsistent (Green et al., 2010 a; Pugliesi et al., 2014), there has been no report on whether *RSAD2* can be used for pregnancy diagnosis. Therefore, the diagnostic value of these three ISGs levels for the detection of pregnancy in different dairy herds still needs to be studied further. Moreover, testing whether a combination of ISGs would improve the efficiency of detection methods also needs be confirmed.

In this study, we systematically tested the expression of *ISG15*, *OAS1,* and *RSAD2* in peripheral blood of Chinese Holstein cows during early pregnancy using single fluorescence real-time quantitative polymerase chain reaction (PCR) and duplex fluorescence real-time quantitative PCR. We evaluated them for early pregnancy diagnosis in dairy cows using the receiver operating characteristic (ROC) curve, and established a formula for early pregnancy diagnosis using these ISGs, thus supplying a more reliable biomarker-based early pregnancy diagnosis strategy for dairy cows.

Material and methods

All animal procedures were approved by The Hubei Province for Biological Studies Animal Care and Use Committee. Protocols were carried out in accordance with Hubei Provincial Regulation on Administration of Laboratory Animals (10/1/2005).

Study 1

Heifers with similar body condition were selected from the dairy farm of Wuhan Huierkang Yangzi River Dairy Co. Ltd., China. After the estrus detection, AI was carried out; 3 mL blood samples were collected from the tail vein on days 0, 14, 18, 21 and 28 after AI (n=19) using $EDTAK_2$ automatic quantitative venous blood collection tube (Wuhan Zhiyuan Medical Treatment Technology Co., Ltd., China). The blood samples were transported to the laboratory within 2 h and RNA was extracted immediately. Whole blood total RNA was extracted using RNAprep pure total RNA extraction kit (Tiangen Biochemical Technology [Beijing] Co. Ltd., China). RNA concentration and purity were analyzed and calculated using NanoDrop 2000 ($OD_{260/280}$ = 1.9–2.1); RNA integrity was analyzed with 1.2% agarose gel electrophoresis; and qualified samples were aliquoted and stored at −80°C. Reverse transcription was carried out using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania). The reaction consumed 500 ng RNA, and the total volume of the reaction was 50 μL.

Rectal palpation was performed 50–60 days after AI; the cows were divided into 11 pregnant cows and 8 non-pregnant cows. Two cows returned to next estrus around day 21 after AI and were remated, and therefore were not analyzed on day 28 after first AI. Fluorescence quantitative PCR was used to detect the expression levels of *ISG15*, *OAS1,* and *RSAD2* in PBL of the pregnant and non-pregnant cows. Fluorescence quantitative PCR was carried out using SYBR Green Mix (TOYOBO Life Sciences, Shanghai, China), which included 10 μL 2 × SYBR Green, 0.5 μL (10 μmol/L) forward and reverse primers, 0.3 μL cDNA template, and addition of

deionized distilled water, to a final volume of 20 µL. Bovine *ISG15*, *OAS1* and the internal reference gene β-actin (*ACTB*) primers were used as described previously (Green et al., 2010 a; Gifford et al., 2007). The primers of *RSAD2* were designed by Primer 5.0 and the specific primer information shown in Table 1. The sample thermal cycling parameters: 95°C for 60 s; 95°C for 15 s, annealing temperatures (see Table 1) for 15 s, and 72°C for 30 s for 40 cycles. For the melting curve and signal acquisition, the fluorescence quantitative PCR products were incubated for 10 s at each step with an increase in temperature of 0.5°C from 58°C to 95°C in each cycle. Each sample was repeated three times and quantitative PCR was performed using a Roche LightCycler 480 real-time PCR instrument (Roche Molecular Systems, Pleasanton, CA, USA).

Table 1. Primers used for fluorescence quantitative PCR

Targets	Primer sequences(5'–3')	Tm/°C	Product size/bp
ISG15	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT	62	87
OAS1	F: ACCCTCTCCAGGAATCCAGT R: GATTCTGGTCCCAGGTCTGA	60	199
RSAD2	F: GCTGAAAGAAGCAGGTATGGA R: CGTACTTCTGGAACCACCTCT	58	176
ACTB	F: CTGGACTTCGAGCAGGAGAT R: GGATGTCGACGTCACACTTC	58–62	208

The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. The expression of each gene at day 0 was set to 1, and the expression at other timepoints was relative to the expression level at day 0. The data were shown as mean ± standard error. The difference in gene expression between pregnant and non-pregnant heifers at different times was analyzed using Student's *t*-test, statistical significance was assigned as $P \leq 0.05$.

Study 2

In order to confirm whether the ISGs were suitable for pregnancy diagnosis, blood samples of 19 pregnant heifers and 17 non-pregnant heifers were collected on day 0 and day 18; total blood RNA treatment and fluorescence quantitative PCR analysis of *ISG15*, *OAS1*, and *RSAD2* was carried out using the same methods as study 1.

The expression levels of *ISG15*, *OAS1*, and *RSAD2* were analyzed separately, or in combination, to evaluate the diagnostic effects in early pregnancy. The sensitivity and specificity of each method were calculated by Statistical Product and Service Solution (SPSS) version 19.0 through analysis of ROC curves. In multi-gene diagnosis, the logistic regression equation was obtained by Binary Logistic in SPSS, and the new variables including individual detection probability were generated in the SPSS working data table. Sensitivity was the probability that diagnosis was positive, which was identified as pregnant by rectal palpation; Specificity was the probability that diagnosis was negative, which was identified as non-pregnant by rectal palpation.

Study 3

According to Study 2, the ISG combination with the optimal diagnostic value was used to design duplex quantitative PCR primers and probes using the TaqMan probe technique. Primers and probes were designed using Beacon Designer 7 (Table 2), and synthesized by Takara (Dalian) Co. Ltd. (China).

Table 2. Primers and TaqMan probes used for duplex fluorescence quantitative PCR

Targets	Primers/probes	Sequences (5'–3')	Tm/°C	Length/bp	Fluorescent label probe
ISG15	*ISG15*-152-F	CATCGCCCAGAAGATCAATGTG	60	156	5' FAM, 3'Eclipse
	ISG15-307-R	ACCAGGATGGAGATGCAGTTC	60		
	ISG15-275-probe	CAGGACGGTGCTGCCAGCTCTCA	69		
RSAD2	*RSAD2*-271-F	CACCAGCGTCAATTACCACTTC	60	121	5' FAM, 3'Eclipse
	RSAD2-391-R	CAGCATCAGCAGACCTTTCTTG	60		
	RSAD2-368-probe	CCTCCTCCAGCGGCAGCACGAAG	69		
ACTB	*ACTB*-526-F	GTGCCCATCTATGAGGGGTAC	60	169	5' HEX, 3'Eclipse
	ACTB-694-R	CGTAGCAGAGCTTCTCCTTGA	60		
	ACTB-564-probe	CCTGCGTCTGGACCTGGCTGGC	69		

cDNA samples used for testing the primers in Table 2 were arbitrarily selected from the study 2. The PCR working solution contained 10 μL Premix Taq, 0.2 μL (10 μmol/L) forward and reverse primers, 0.6 μL cDNA template and deionized distilled water. The following times and temperatures were used: 94°C for 3 min; 94°C for 30 s, 61°C for 30 s and 72°C 25 s for 30 cycles; 72°C for 10 min. PCR products were analyzed with 1.2% agarose gel electrophoresis.

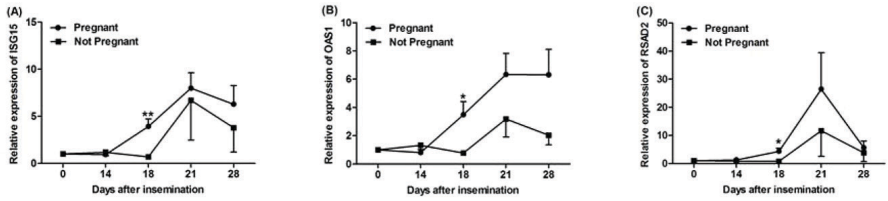
Six cDNA samples were arbitrarily selected from Study 2. Each sample was detected both in the single fluorescence quantitative PCR and in the duplex fluorescence quantitative PCR, completed on the same reaction plate. The single fluorescence quantitative PCR was used for detecting the expression of the target genes (*ISG15* or *RSAD2*) and control gene (*ACTB*), respectively; the PCR system included 10 μL 2× Premix Ex Taq Mix, 0.2 μL forward and reverse primers (10 μmol/L), 0.4 μL probe (10 μmol/L), 0.6 μL cDNA template, and deionized distilled water for a final volume of 20 μL. The duplex fluorescence quantitative PCR was designed to detect the expression of the target and control genes simultaneously in the same reaction well; the PCR system was composed of 10 μL 2× Premix Ex Taq Mix, 0.2 μL forward and reverse primers (10 μmol/L), 0.4 μL probe of the target gene and control gene, 0.6 μL cDNA template, and deionized distilled water for a final volume of 20 μL. The amplification was performed using 95°C 30 s; 95°C 15 s, and 61°C 30 s for 40 cycles with a CFX Connect fluorescent quantitative PCR Detection System (Bio-Rad, Hercules, CA, USA). The fluorescence signals were acquired at the annealing and extension phase of each cycle. Real-time analysis software was used to calculate the cycling threshold (Ct) for each sample. The ΔCt of the target genes were compared between the single and duplex PCR assays.

All the samples in Study 2 were tested using duplex fluorescence quantitative assay when the reaction system was well established. The sensitivity and specificity of pregnancy diagnosis for using *ISG15* combined with *RSAD2* gene expression were analyzed by ROC analysis, and the results were compared with SYBO Green single quantitative assays.

Results

Expression of *ISG15*, *OAS1*, and *RSAD2* in peripheral blood of heifers during early pregnancy

Overall, the expression of *ISG15*, *OAS1*, and *RSAD2* in peripheral blood showed an upward trend within 21 days after AI (Figure 2). From 14 to 18 days after AI, the expression levels of *ISG15*, *OAS1*, and *RSAD2* increased in the peripheral blood of pregnant cows. However, the expression of *ISG15*, *OAS1*, and *RSAD2* was unchanged in the non-pregnant cows. On day 18 after AI, expression of *ISG15* in pregnant cows was significantly higher than that in non-pregnant cows ($P<0.01$), and expression of *OAS1* and *RSAD2* was significantly higher than that of non-pregnant cows ($P<0.05$). Although the expression of the three genes in pregnant cows was higher than non-pregnant cows on day 21 after AI, the difference was not significant ($P>0.05$).



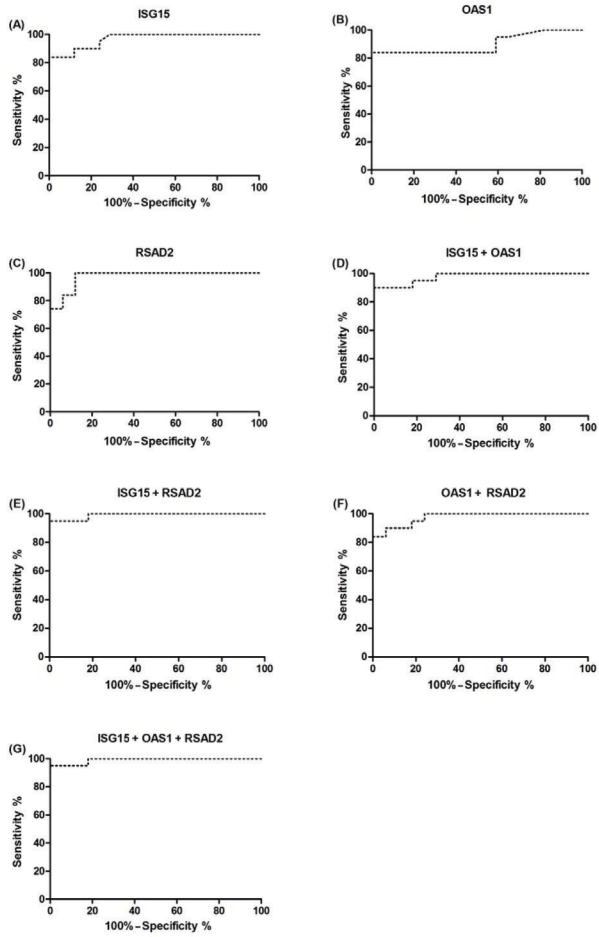
(A) relative expression of *ISG15*. (B) relative expression of *OAS1*. (C) relative expression of *RSAD2*. **Significance level of 0.01; *Significance level of 0.05.

Figure 2. Relative gene expressions of bovine *ISG15, OAS1,* and *RSAD2* in peripheral blood of heifers during early pregnancy

Effectiveness evaluation of *ISG15*, *OAS1*, and *RSAD2* levels for early pregnancy diagnosis of dairy cows

The ROC curves for the diagnosis of early pregnancy in dairy cows were established for the expression of *ISG15*, *OAS1*, and *RSAD2* on day 18 after AI (Figure 3). The area under the ROC curve (AUC) representing the diagnosis effectiveness of pregnancy using ISGs expression showed that $AUC_{ISG15+OAS1+RSAD2} = AUC_{ISG15+RSAD2} > AUC_{ISG15+OAS1} = AUC_{OAS1+RSAD2} = AUC_{RSAD2} > AUC_{ISG15} > AUC_{OAS1}$. The cutoff value was the threshold determined by the maximum value of the Youden index (YD) (Greiner et al., 2000). When the cut-off value was 1.152, the sensitivity of *RSAD2*

for early pregnancy diagnosis was 100%, but the specificity was 88.2%. When the cut-off value for univariate *ISG15*, *OAS1,* and multivariate *ISG15* + *OAS1*, *ISG15* + *RSAD2*, *OAS1* + *RSAD2* and *ISG15* + *OAS1* + *RSAD2* were 2.190, 1.815, 0.651, 0.680, 0.831 and 0.615, respectively, the specificity of the early pregnancy diagnosis methods was 100% for all. The groups of *ISG15* + *RSAD2* and *ISG15* + *OAS1* + *RSAD2* had the highest accuracy (97.2%). Considering the sensitivity, specificity, accuracy and simplicity of the diagnostic method, the combination of *ISG15* and *RSAD2* was best to establish a duplex quantitative PCR assay system.



(A) ROC curve for *ISG15*. (B) ROC curve for *OAS1*. (C) ROC curve for *RSAD2*. (D) ROC curve for *ISG15+OAS1*. (E) ROC curve for *ISG15+ RSAD2*. (F) ROC curve for *OAS1+ RSAD2*. (G) ROC curve for *ISG15+OAS1+ RSAD2*.

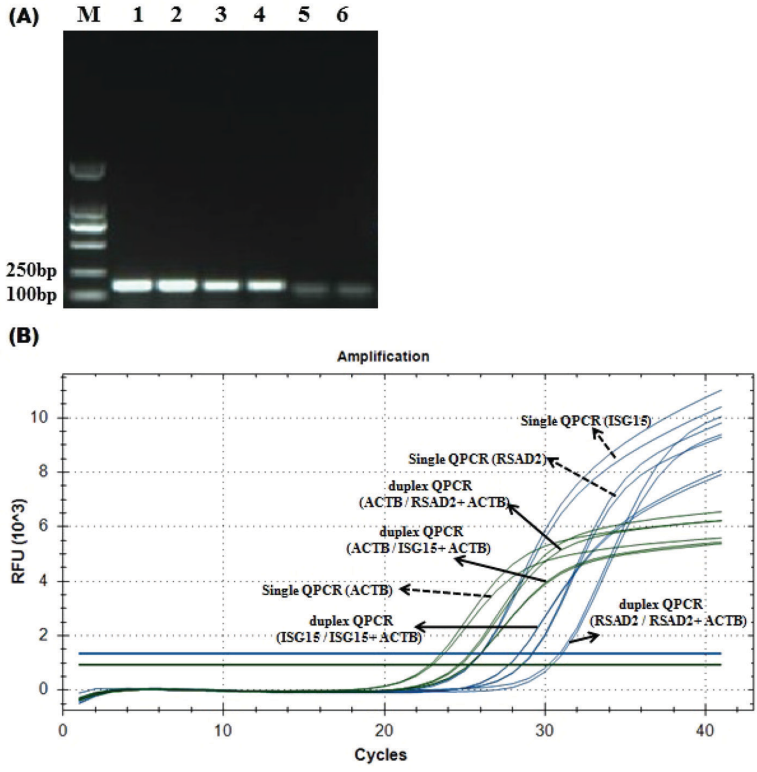
Figure 3. Receiver operating characteristic (ROC) curves for single or combined interferon stimulated genes in pregnancy diagnosis

Table 3. Ct values of *ISG15* and *RSAD2* with single and duplex fluorescence quantitative PCR assays

Samples	Single fluorescence quantitative PCR					Duplex fluorescence quantitative PCR					
	ISG15	*RSAD2*	*ACTB*	ΔCt (*ISG15*)	ΔCt (*RSAD2*)	*ISG15* (*ISG15*+*ACTB*)	*ACTB* (*ISG15*+*ACTB*)	*RSAD2* (*RSAD2*+*ACTB*)	*ACTB* (*RSAD2*+*ACTB*)	ΔCt (*ISG15*)	ΔCt (*RSAD2*)
1	26.02	29.19	22.91	3.11	6.28	28.37	25.24	30.98	24.65	3.13	6.33
2	26.36	29.73	21.41	4.94	8.32	28.09	23.14	31.14	23.11	4.95	8.03
3	26.83	29.42	19.56	7.27	9.86	29.13	22.36	31.65	22.19	6.77	9.45
4	33.18	35.55	26.11	7.07	9.44	33.97	26.83	36.22	26.92	7.15	9.29
5	31.17	31.67	23.00	8.17	8.67	31.41	23.29	31.96	23.61	8.13	8.35
6	29.18	32.27	21.41	7.77	10.85	30.31	23.38	33.47	23.84	6.93	9.62

Establishment of a duplex quantitative PCR assay system

Expression of *ISG15* and *RSAD2* in peripheral blood of dairy cows was measured by single and duplex quantitative PCR simultaneously in the same quantitative PCR plate (Table 3 and Figure 4). Table 3 showed that duplex quantitative PCR detection for the same sample gave stable Ct values of *ACTB* for the combination of *ISG15* or *RSAD2* with *ACTB,* the difference of the Ct values of *ACTB* between the two combinations was under 0.6 which indicates the repeatability of this experiment. For the same sample, Ct values of the same target gene (*ISG15* or *RSAD2*) detected in duplex quantitative PCR were 0 to 2.35 higher than the single quantitative PCR. However, comparison between duplex and single quantitative PCR results showed that ΔCt value of *ISG15* varied from −0.84 to 0.08 and ΔCt value of *RSAD2* varied from −1.23 to 0.05, indicating that the duplex quantitative PCR was highly specific for discrimination between the target gene and control genes. Therefore, the cross-talk between target gene and control gene was small, and the duplex quantitative PCR system was stable.



(A) Agarose gel electrophoresis results for the amplifications: line 1–2, *ISG*15; line 3–4, *OAS*1; line 5–6, *RSAD*2. (B) Amplification curves.

Figure 4. Amplification plots for Single and Duplex fluorescence quantitative PCR

Table 4. Sensitivity, specificity, cutoff value, Youden index (YD) and area under the curve (AUC) for determining pregnancy status on day 18 post AI by the expression of *ISG15* and *RSAD2* with duplex fluorescence quantitative PCR using PBL

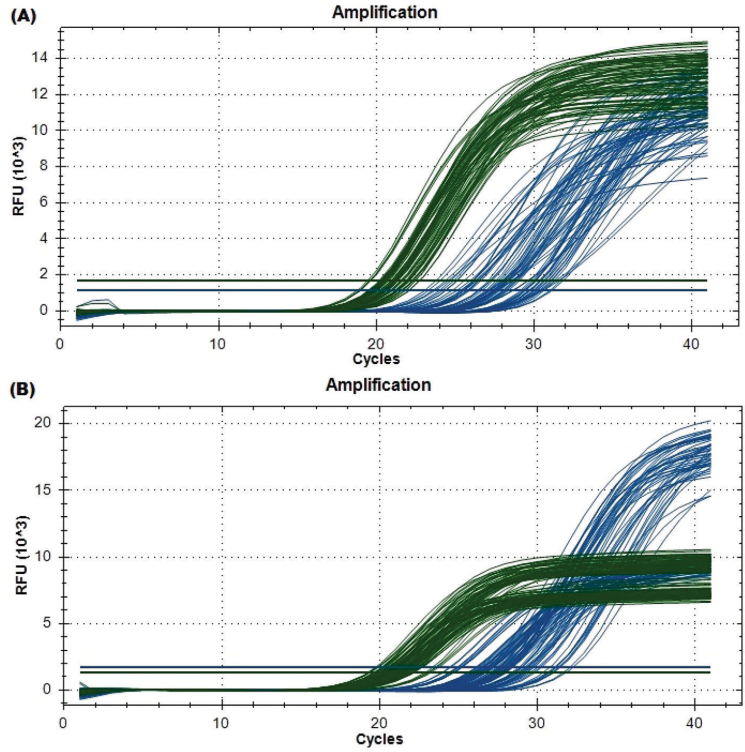
Genes	*ISG15*	*RSAD2*	*ISG15+ RSAD2*
Number of cows	36	36	36
AUC	0.994	0.935	0.991
YD	0.947	0.777	0.947
Cutoff value	2.227	1.512	0.620
Sensitivity	94.7	89.5	94.7
Specificity	100	88.2	100

Accuracy of pregnancy diagnosis using duplex fluorescence quantitative PCR

Amplified blood RNA sample results were obtained by double fluorescence quantitative PCR for pregnant cows (n = 19) and non-pregnant cows (n = 17) as diagnosed by rectal palpation as shown in Figure 5. Expression of the target gene *mRNA* in the sample was calculated by $2^{-\Delta\Delta Ct}$ method. The ROC curve was established using *ISG15* and *RSAD2* in combination with early pregnancy diagnosis, and the sensitivity and specificity shown in Table 4. When the cutoff value was 2.227, the sensitivity of using *ISG15* gene results alone for pregnancy diagnosis by duplex quantitative PCR system was 94.7%, which was higher than that using single quantitative PCR. However, at the cutoff value of 1.402, the sensitivity of *ISG15* gene alone was 100% and the specificity was 88.2%. In contrast, the sensitivity of using *RSAD2* alone in duplex quantitative PCR was lower than that of single quantitative PCR. Additionally, the sensitivity and specificity of the combination of the two genes were 94.7% and 100%, respectively, which were consistent with the results of single quantitative PCR. The cut-off value (0.620) was also close to that of single quantitative PCR. Table 5 showed results of the logistic regression equation: $P=1/[1+e^{-(-7.014+3.518X1-0.155X2)}]$ (X_1 represents the expression of *ISG15* by duplex fluorescence quantitative PCR, X_2 represents the expression of *RSAD2* by duplex fluorescence quantitative PCR). When the simultaneous expression of *ISG15* and *RSAD2* in a sample simultaneously satisfies $P \geq 0.620$, the individual should be diagnosed as pregnant.

Table 5. The regression coefficient, standard error, and P-value of the covariates *ISG15* and *RSAD2* based on the logistic mode

Covariates	Regression coefficient	Standard error	P-value
ISG15	3.518	1.933	0.069
RSAD2	–0.155	1.673	0.926
Constant	–7.014	2.818	0.013



(A) pregnant cows. (B) non-pregnant cows.

Figure 5. Amplification curves for duplex fluorescence quantitative PCR of *ISG15* and *RSAD2* gene in pregnancy diagnosis

Discussion

Diagnosis of early pregnancy helps to identify open cows and guides re-breeding before the next ovulation. Thus, accurate and simple early pregnancy diagnosis technology has attracted much attention. Recently, detection of ISG expression in peripheral blood leukocytes was introduced as a new method for the detection of pregnancy. Green et al. (2010 b) successfully used the ratio of the expression of *OAS1* in PBL during the first 18 days after fixed-time AI and its expression at 3 days before fixed-time AI to identify non-pregnant cows. The identified non-pregnant cows were injected with $PGF_{2\alpha}$ at day 19 and injected with GnRH at day 21 followed by the second fixed-time artificial insemination (TAI). The successful pregnancy rate of the non-pregnant cows after AI was slightly higher than that of natural estrus, and the insemination interval was effectively shortened. In contrast, higher pregnancy rate or reduced open days were not observed if the PAGs ELISA kit was used to identify the non-pregnant cows at day 28 after AI with simultaneous estrus (Sinedino et al.,

2014). This research showed that the method of pregnancy diagnosis based on ISGs expression in peripheral blood leukocytes can be used to effectively improve the reproductive efficiency of dairy cows. Therefore, selection of the biomarker gene for the diagnosis of early pregnancy has attracted attention in the dairy industry.

Table 6. Expression levels of bovine *ISG15* gene in peripheral blood of heifers during early pregnancy

Group	Number of the cows	Days after insemination				
		0 d	14 d	18 d	21 d	28 d
Pregnant cows	100480	1.00	1.09	6.08	12.85	17.23
	100482	1.00	0.96	8.51	13.90	12.97
	100253	1.00	0.57	1.15	3.13	1.55
	10128	1.00	0.70	1.54	2.97	1.14
	100547	1.00	1.69	2.68	5.31	4.25
	100429	1.00	1.87	8.24	17.96	8.09
	1072	1.00	0.46	4.00	6.65	3.86
	1073	1.00	0.39	1.00	2.59	0.36
	1074	1.00	0.30	2.76	4.21	1.07
	8034	1.00	1.28	2.92	5.05	17.23
	8014	1.00	1.03	4.29	13.24	1.35
Non-pregnant cows	100517	1.00	0.30	0.31	35.92	16.56
	110001	1.00	0.82	0.64	0.94	2.20
	100511	1.00	1.41	0.86	4.65	1.30
	100434	1.00	3.32	0.81	1.44	1.52
	9067	1.00	1.02	1.00	1.48	0.49
	8019	1.00	0.27	0.70	1.40	0.58
	100567	1.00	1.86	0.80	6.73	5.90
	100334	1.00	0.48	0.38	1.09	43.81

Bold and italic number of the cows means it returns to next estrus around 21 d (the same as below).

By using microarray technology, Kizaki et al. (2013) found that expression of *RSAD2* in peripheral blood leukocytes at day 21 after AI was 2.33 times higher than that at day 0 in delivered cows, but the expression level did not change significantly at day 14. However, Green et al. (2010 a) did not identify differences in *RSAD2* expression by comparing transcriptomic differences in PBL between days 15 and 18 after AI in primiparous cows. Previous studies have focused on the change in *RSAD2* expression during different gestation periods. However, whether *RSAD2* is differentially expressed between pregnant and non-pregnant cows is not clear. In the present study, expression of *RSAD2* in leukocytes at days 0, 14, 18, 21 and 28 after AI of heifers was tested. Expression level of *RSAD2* in the peripheral blood during pregnancy showed an upward trend, which was significantly higher than that in the non-pregnant cows at day 18 after AI. This suggested that *RSAD2* could be used as a biomarker for pregnancy diagnosis. Nevertheless, there was no significant differ-

ence in the expression of *RSAD2* at day 21 between the pregnant and non-pregnant cows. It is possible that non-pregnant cows were almost all (90%–100%) successfully fertilized. The majority of embryonic loss occurs in the first 3 weeks, during the period of maternal pregnancy recognition, and most of the non-pregnant cows did not present estrous behaviour following day 21 after AI. Therefore, the *RSAD2* expression increased from day 18 to day 21 in non-pregnant cows, while the expression level declined from day 21 to day 28 (Table 6, Table 7 and Table 8) because the non-pregnant cows are preparing for the second estrus, and actually returned to next estrus later than day 28. Further analysis using ROC curves showed the sensitivity was 100%, implying that the risk of misdiagnosing pregnant cows as non-pregnant was low. However, it should be noted that because of the low specificity, it could increase the possibility of misdiagnosing non-pregnant cows as pregnant.

Table 7. Expression levels of bovine *OAS1* gene in peripheral blood of heifers during early pregnancy

Group	Number of the cows	Days after insemination				
		0 d	14 d	18 d	21 d	28 d
Pregnant cows	100480	1.00	1.46	6.88	9.85	17.15
	100482	1.00	1.09	6.62	18.55	9.25
	100253	1.00	0.91	1.83	2.13	1.88
	10128	1.00	0.64	9.96	5.07	1.59
	100547	1.00	0.68	0.69	0.71	5.74
	100429	1.00	0.80	3.49	6.34	6.32
	1072	1.00	0.60	2.10	6.31	4.64
	1073	1.00	0.41	0.73	2.56	0.37
	1074	1.00	0.37	2.50	5.25	2.94
	8034	1.00	0.53	0.78	3.61	17.35
	8014	1.00	1.34	2.83	9.34	2.29
Non-pregnant cows	100517	1.00	0.23	0.14	11.77	5.15
	110001	1.00	1.12	1.13	1.38	2.54
	100511	1.00	2.50	0.64	2.71	1.09
	100434	1.00	2.50	0.70	1.33	1.00
	9067	1.00	0.94	0.79	3.55	0.69
	8019	1.00	0.37	0.69	1.30	1.74
	100567	1.00	1.50	0.92	2.51	8.53
	100334	1.00	1.42	1.23	0.90	53.32

There are many studies focusing on using *ISG15* and *OAS1* as biomarkers to diagnose early pregnancy of dairy cows (Han et al., 2006; Green et al., 2010 a; Pugliesi et al., 2014; Green et al., 2010 b; Mauffré et al., 2016), yet the results are debatable. Shirasuna et al. (2012) showed that expression of *ISG15* and *OAS1* in polymorpho-

nuclear leukocytes (PMNs) of pregnant cows on day 5 after AI was significantly higher than that in non-pregnant cows, However, the reliability of pregnancy diagnosis using ISGs in PMNs is still unclear. Green et al. (2010 a) reported that the sensitivity and specificity of pregnancy diagnosis in heifers on day 18 after AI using *OAS1* expression in PBL were both 100%. On the other hand, the expression level of *OAS1* in both granulocytes and whole blood was not significantly different in pregnant cows compared to those in non-pregnant cows on days 20 to 22 after AI (Yoshino et al., 2018). In the present study, the expression of *ISG15* and *OAS1* in peripheral blood of pregnant cows on day 18 after AI was significantly higher than those in non-pregnant cows. However, the threshold values and sensitivity of these two genes for pregnancy diagnosis were not consistent with previous reports, which may have been due to the study group and the sample processing method. First, the animals used in this study were from commercial dairy farms. Although disease prevention and breeding were according to standard procedures, abnormal uterus environment, such as hemorrhage, pus liquid after AI, was not observed. It was difficult to ensure that there were no bacteria, viruses or other pathogens in the actual production conditions. ISGs could be regulated by the other interferon, especially in some pathological conditions (Figure 1), indicating that animal health status will affect the application of ISGs in the diagnosis of early pregnancy. Previous studies have confirmed that *ISG15* expression is significantly increased in virus-infected sheep (Rodrigues Hoffmann et al., 2013). Secondly, in some studies, whole blood samples were collected using EDTA tubes. EDTA does not contain nucleic acid stabilization factors, therefore, gene expression, especially expression of ISGs and some other cytokine genes, can vary in the process of cell separation (Sheridan et al., 2012). Although the blood sample collection method may not be the main factor affecting the application of ISGs in diagnosis of early pregnancy, it may influence the diagnostic sensitivity and specificity (Mauffré et al., 2016). Moreover, as a result of differences in PCR tests, the reproducibility of pregnancy diagnosis using specific cut-off values in different herds and laboratories is poor (Pugliesi et al., 2014).

The key to an innovative early pregnancy diagnosis method is to distinguish pregnancy from non-pregnancy, because its accuracy profoundly affects the application of this technology in research and breeding procedures (Giordano et al., 2013). Therefore, we combined *RSAD2*, *ISG15*, and *OAS1* in this study. Results showed that the diagnostic accuracy using the combination of *RSAD2* and *ISG15* levels was 97.2%; this was higher than that using a single ISG, and was consistent with the results of Pugliesi et al. (2014). In practice, although diagnosis based on multiple ISGs improves accuracy, it also increases the workload as well as the cost. Multiple quantitative PCRs are gradually being used for clinical detection but, in bovine studies, they are mainly applied for detection of pathogenic bacteria (Selim et al., 2014; Soltan et al., 2015; Shridhar et al., 2016). The advent of multiple quantitative PCR technology provides a new method for the use of ISGs in pregnancy diagnosis. The economic and widely used TaqMan technology was implemented, and a duplex fluorescence quantitative PCR technology established by optimization of primers and probe concentrations. The duplex fluorescence quantitative PCR can simultaneously detect target genes (*ISG15* or *RSAD2*) and reference gene (*ACTB*) in one reaction

system; two target genes (*ISG15* or *RSAD2*) also can be detected in one PCR reaction plate. Our results showed that duplex and single fluorescence quantitative PCR technologies had consistent ΔCt values, suggesting that these two types of fluorescence signal do not interfere with each other. In addition, the present study showed that the sensitivity, specificity and critical value of early pregnancy diagnosis in dairy cows using duplex fluorescence quantitative PCR technology were consistent with that using single fluorescence quantitative PCR technology. This indicates that PCR detection systems based on *ISG15* and *RSAD2* were successfully established.

Table 8. Expression levels of bovine *RSAD2* gene in peripheral blood of heifers during early pregnancy

Group	Number of the cows	Days after insemination				
		0 d	14 d	18 d	21 d	28 d
Pregnant cows	100480	1.00	2.22	4.46	23.53	26.35
	100482	1.00	0.85	4.12	19.70	12.45
	100253	1.00	0.53	1.23	3.17	1.06
	10128	1.00	0.85	3.38	4.09	1.01
	100547	1.00	0.85	1.35	4.32	3.94
	100429	1.00	2.05	12.01	32.82	8.57
	1072	1.00	0.52	2.76	5.22	1.82
	1073	1.00	4.14	9.92	27.03	2.26
	1074	1.00	0.51	3.33	6.56	1.18
	8034	1.00	0.48	1.52	151.87	0.25
	8014	1.00	1.11	4.01	12.73	3.72
Non-pregnant cows	100517	1.00	0.63	0.65	75.24	19.12
	110001	1.00	1.00	0.80	0.76	1.45
	100511	1.00	1.04	0.82	1.57	0.82
	100434	1.00	0.73	0.64	0.74	0.70
	9067	1.00	1.51	1.08	6.88	0.62
	8019	1.00	0.50	0.65	1.77	0.13
	100567	1.00	0.50	0.96	6.12	4.12
	100334	1.00	0.72	0.39	0.39	30.91

This study reported early pregnancy diagnosis of dairy cows using TaqMan duplex quantitative PCR technology. The detection time of this new technique is shortened by half, with savings on detection reagents and consumables. Therefore, the detection efficiency was effectively improved, promoting this application in production and scientific research. However, the sensitivity of early pregnancy diagnosis using duplex fluorescence quantitative PCR has not yet reached 100%. It is still possible to misdiagnose pregnant cows as non-pregnant, thus requiring further improvement. The sensitivity of pregnancy diagnosis using *ISG15* or *RSAD2* obtained from duplex quantitative PCR amplification alone may change due to changes in the probe reac-

tion solution and system. However, the sensitivity can still reach 100% if *ISG15* measured from duplex quantitative PCR amplification is used alone with a cut-off value of 1.402 (data not shown). Besides accurate diagnosis of pregnancy in dairy cows, the diagnostic marker gene needs to have low false-positive or false-negative rates (Balhara et al., 2013). We suggest that when expression of ISGs is used to diagnose pregnancy of dairy cows using duplex fluorescence quantitative PCR, *ISG15* should first be used alone to identify pregnant cows, followed by identification of non-pregnant cows using *ISG15* and *RSAD2* in combination to ensure the accuracy is 100%. The remaining cows can then be categorized as suspected pregnant or non-pregnant and should be diagnosed using other methods. In the future, we need to increase the sample size of the cows to obtain the optimal gene expression threshold through further verification.

In conclusion, *ISG15*, *OAS1*, and *RSAD2* can all be used as biomarkers for the diagnosis of early pregnancy in cows. Specifically, *RSAD2* has the highest sensitivity, while the combination of *ISG15* and *RSAD2* has the highest accuracy with single fluorescence quantitative PCR. Based on our duplex fluorescence quantitative PCR detection method for early pregnancy diagnosis in dairy cows, we can identify the pregnant cows using *ISG15* alone, followed by identification of non-pregnant dairy cows using *ISG15* and *RSAD2* in combination. This method is expected to improve two-way identification of both non-pregnant and pregnant cows.

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