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The efficiency of interferon stimulated gene 15 expression in peripheral blood for early pregnancy prediction in Awassi ewes

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Abstract

This study was designed to identify the efficiency of interferon-stimulated gene 15 (ISG15) expression for early pregnancy detection in Awassi ewes. A total of 10 ewes were enrolled: 3 carrying twins, 4 carrying single pregnancies, and 3 non-pregnant. Blood samples were collected on 0, 6, 15, and 25 days of gestation to measure ISG15 expression levels using quantitative real-time PCR (qRT-PCR). The results of the relative expression values of ISG15 in pregnant and non-pregnant ewes on 0, 6, 15, and 25 days of pregnancy were 0.044±0.012 vs. 0.049±0.009, 0.203±0.083 vs. 0.201±0.093, 1.839±0.594 vs. 0.292±0.136, and 0.583±0.067 vs 0.200±0.051, respectively. In both twin and single pregnant ewes, the relative expression values of ISG15 were increased significantly ($P<0.05$) on 15 days of pregnancy, after that the values declined significantly ($P<0.05$) on day 25. No significant differences in ISG15 expression were observed between ewes carrying twins and those carrying single fetuses. Receiver operating characteristic (ROC) curve analysis showed that the area under the curve (AUC) on days 0 and 6 of pregnancy was 0.405, whereas on days 15 and 25 it was 0.952 and 1.00, respectively. The accuracy, specificity, sensitivity, and positive and negative predictive values of ISG15 expression on days 15 and 25 of pregnancy were 90%, 100%, 85.7%, 100%, and 75%, and 100%, 100%, 100%, 100%, and 100%, respectively. In conclusion, ISG15 expression demonstrated high accuracy as an early pregnancy marker in Awassi ewes, with greater diagnostic reliability on day 25 compared to day 15 of gestation.

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Introduction

Accurate pregnancy detection is critical for efficient sheep herd management. Numerous methods of pregnancy diagnosis have been applied, including radiography, hematological parameters, progesterone assay, estrone sulphate assay, ovine pregnancy specific protein B assay, pregnancy-associated glycoprotein assay, A-mode and Doppler ultrasonic techniques, transrectal and transabdominal B-mode real time ultrasonography, and reproductive genes expression (1-7). In mammals, the peri-implantation phase is essential for embryonic survival and successful pregnancy. During this period, which includes the development of the embryo/fetus and extraembryonic membranes, effective implantation and placentation require reciprocal communication among the ovary, conceptus, and endometrium (8). In sheep, the spherical blastocyst elongates into a tubular, then filamentous shape, forming a conceptus. Throughout this stage, mononuclear trophectodermal cells produce and release interferon tau (IFN-tau), with peak secretion occurring between days 14 and 16 (9). The IFN-tau directly affects the epithelium of endometrium and superficial ductal glandular epithelium in the uterus of ewes. This suppresses the transcription of the genes encoding the

oxytocin receptor and estrogen receptor alpha, which stops the production of prostaglandin F2α (10). IFN-tau also induces the transcription of interferon-stimulated gene 15 (ISG15), which plays a key role in regulating endometrial receptivity at implantation. This process is crucial for embryonic growth, survival, and development (11). The expression ISG15 in uterine endometrium of pregnant sheep was recorded significantly increased during the first and two days (12,13), peaked during 13-19 day of pregnancy and decline to basal level on day 21 of pregnancy (14). ISG15 have been identified as early pregnancy diagnosis markers with variable effectiveness in cattle (15,16), buffalo (17,18), goats (19) and sheep (13,14,20). In sheep, the previous studies indicated that IFN-tau that produced by embryo released from the uterus into the uterine vein (13) to induce ISGs through an endocrine mechanism in extra uterine tissues such as peripheral blood leukocytes (21), corpus luteum (13), bone marrow (22), thymus (23), lymph nodes (24), also in the liver (25), spleen (26) and thyroid (27). Therefore, the expression of ISG15 was evaluated in these tissues for early detection of pregnancy in ewes.

Although ISG15 expression has been measured in sheep to confirm pregnancy, no studies to date have specifically reported its diagnostic accuracy. Therefore, the present research was conducted to determine the accuracy, sensitivity, and specificity of ISG15 expression for early pregnancy detection in Awassi ewes.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq (No. P.G/1662 on 11/9/2024).

Animals

The current study was part of the PhD research project (only 10 ewes out of 24 ewes were used), which was carried out in the sheep experimental farm of the Office of Agricultural Researches / Department of Agricultural Researches / Nineveh during the period between June and December 2023. The PhD research project was designed to use twenty-four mature non-pregnant Awassi ewes, and six Awassi rams for heat detection and breeding (which that were separated from the ewes for six weeks before treatments). The animals were placed in semi-opened shade shelter and provided with concentrate feed mixture and roughages and fresh water was available ad libitum. Different protocols were applied for ewe's estrus synchronization to ensure obtaining single and twin pregnancy in ewes. The ewes were inseminated naturally at the end of estrus synchronization protocols by rams and the date of insemination was considered to be zero day of pregnancy.

Blood collection

Blood samples were collected on days 0, 6, 15 and 25 of pregnancy from jugular vein. A portion of each sample was added to a collecting tube containing 400µL of TRIzol reagent; blood-TRIzol should have a final volume of 1 mL, which was promptly frozen at -20°C for quantitative Real-Time PCR (qRT-PCR) for determining the ISG15 Expression.

Ultrasonography

A portable real-time B-mode ultrasonographic device (KAIXIN, KX5100, China) was used for pregnancy confirmation on day 40. Transabdominal ultrasonography was performed with a 3.5 MHz convex array transducer, following standard procedures for pregnancy detection in sheep and goats (28,29). Based on these scans, 10 ewes (3 twin-pregnant, 4 single-pregnant, and 3 non-pregnant) were included in the current analysis.

The primers preparation

The stock solution of primers was prepared by dissolving of lyophilized primers in free double distilled water to give a final concentration of 100 pmol/µl. It was stored at -20°C for later use in preparing of 10 pmol/µl working primer suspension (addition of 10 µl stock solution to 90 µl double distilled water to get 100 µl working primer suspension).

RNA Extraction and qRT-PCR Assay

Primers of Real-time Polymerase Chain Reaction of ISG15 gene Two primers (Macrogen/ South Korea) were designed to amplify the mRNA of ovine ISG15 gene, which is part of interferon stimulated genes. Additionally, two primers were utilized to amplify the mRNA of housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase, which derived from Mauffre *et al.* (20). (Table 1).

The blood-TRIzol samples were utilized for qRT-PCR. Following RNA extraction, the total RNA from the blood samples was extracted using TRIzol Reagent, according to TransZol Up Plus RNA Kit TransGen Biotech, China, and the cDNA was synthesized with EasyScript® First-Strand cDNA synthesis Super Mix Transgen, China. GoTaq® qPCR Master Mix (Promega, USA) was employed for qRT-PCR. Two PCR tubes were produced for each sample, one to measure ISG15 gene expression levels and the other to normalize the PCR results (housekeeping gene). The qRT-PCR revealed significant upregulation of ISG genes in the blood cells of pregnant ewes in comparison to non-pregnant ewes. The primers for ISG15 were designed based on Khan *et al.* (21). qRT-PCR amplification was conducted at 94°C for 30 minuts, 94°C for 5 minuts, 60°C for 35 minuts for 45 cycles, and 90°C for 15 minuts for 100 cycles. ISG15 expression was determined using qRT-PCR. qRT-PCR was performed with cDNA which was used with a 25 µl reaction volume as the template.

Table 1: The sequence of primers which were used in current study

Primers	Sequence	Primers sequence 5'- - 3'	Tm (ºC)	GC%
ISG-15 gene	F	CCATGACGGTATCCGAGCTA	61.1	55
	R	GGGCCTCCCTTCAAAAGACA	64	55
GAPDH Reference gene	F	CTCCCAACGTGTCTGTTGTG	61.5	55
	R	TGAGCTTGACAAAGTGGTCG	59.9	50

Analysis of data

The relative expression values of the ISG15 were calculated using the $2^{-\Delta\Delta Ct}$ analysis method (30). The fold change was calculated by the following equations: ΔCT = CT of target gene – CT of reference gene, ΔΔCT = ΔCT of each sample - average control ΔCT, Fold change = $2^{-\Delta\Delta Ct}$. According to the result of conformation transabdominal ultrasonography on day 40 of pregnancy, the result of pregnancy diagnosis in ewes which that achieved by evaluation of the relative expression value of ISG15 were arranged as follows: correct positive diagnosis, incorrect positive diagnosis, correct negative diagnosis, and incorrect negative diagnosis. From these values; the accuracy, sensitivity, specificity, predicted positive value and predicted negative values were calculated (31,32).

Statistical analysis

The relative expression values of ISG15 were reported as mean ± SE. Variations among groups were analyzed using two-way repeated-measures analysis of variance (ANOVA), and significant differences were determined by Duncan's multiple range test. Accuracy, specificity, sensitivity, positive predictive value, and negative predictive value were compared using the Chi-square test. All statistical analysis including the calculation of cut-off point values of relative ISG15 expression in pregnant and non-pregnant ewes were performed by SPSS software (IBM SPSS Statistics, Version 29.0.2.0). The P values equal and less than 0.05 were considered significant.

Results

Transabdominal ultrasonographic confirmation of pregnancy in ten Awassi ewes on day 40 post-insemination indicated that seven ewes were pregnant and three were non-pregnant. Among the seven pregnant ewes, three were carrying twins and four had single pregnancies (Table 2).

Table 2: Results of transabdominal ultrasonographic pregnancy diagnosis in Awassi ewes on day 40 after insemination

Result of diagnosis	Number of ewes
Pregnant-twin	3
Pregnant-single	4
Non-pregnant	3
Total	10

The relative expression of ISG15 that measured in pregnant and non-pregnant ewes on the day of insemination (0 day of pregnancy), as well as on days 6, 15 and 25 of pregnancy were 0.044 ± 0.012 vs 0.049 ± 0.009, 0.203 ± 0.083 vs 0.201 ± 0.093, 1.839 ± 0.594 vs 0.292 ± 0.136 and 0.583 ± 0.067 vs 0.200 ± 0.051, respectively. In pregnant ewes, the relative expression of ISG15 significantly increased (P<0.05) on day 15 of pregnancy compared to days 0 and 6. This value then significantly declined (P<0.05) on day 25 to 0.583±0.067. In non-pregnant ewes, no significant changes were observed in the relative expression of ISG15 throughout the study period. No significant differences were found in the relative expression of ISG15 between pregnant and non-pregnant ewes on days 0, 6 and 25 of pregnancy. However, the relative expression of ISG15 on day 15 of pregnancy was significantly higher (P<0.01) in pregnant ewes compared to non-pregnant ewes (Figure 1).

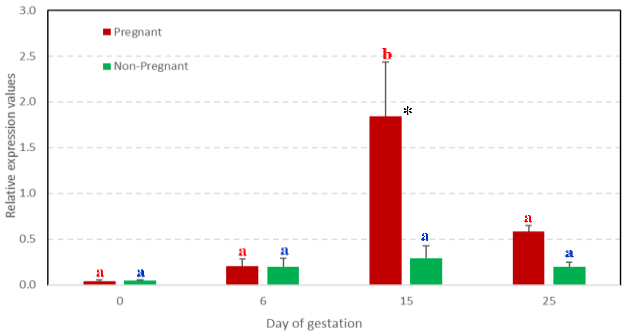

Figure 1: Relative ISG15 expression value in pregnant (n=7) and non-pregnant (n=3) ewes on days 0, 6, 15 and 25 after insemination. [a,b] different small red letters refer to a significant variation between the values of pregnant ewes. [a] small blue letter indicates no significant variation between the values of non-pregnant ewes. * Refers to a significant variation between the values of pregnant and non-pregnant ewes.

Relative expression values of ISG15 which were calculated in twin and single pregnant ewes additional to that of non-pregnant ewes were presented in table 3. In both twin and single pregnant ewes, the relative ISG15 expression values were increased significantly (P<0.05) on day 15 of pregnancy, after that the values were decline significantly (P<0.05) on day 25 of gestation. No significant variance was recorded in relative expression values of ISG15 between the

ewes that have twin pregnancy and those that have single pregnancy over the duration of the study, although the relative ISG15 expression value was greater in twin pregnant ewes.

Table 3: Relative ISG15 expression values (mean ± SE) in twine and single pregnant and non-pregnant ewes on days 0, 6, 15 and 25 after insemination

Pregnancy	Number of ewes	Days of pregnancy			
		0	6	15	25
Twins	3	0.045±0.019 ^{a,A}	0.208±0.089 ^{a,A}	2.034±0.415 ^{a,B}	0.656±0.078 ^{a,A}
Single	4	0.043±0.018 ^{a,A}	0.199±0.142 ^{a,A}	1.693±0.468 ^{a,B}	0.528±0.102 ^{a,A}
Non-pregnant	5	0.049±0.009 ^{a,A}	0.201±0.093 ^{a,A}	0.292±0.136 ^{b,A}	0.200±0.051 ^{a,A}

^{a,b} different red small letters refer to a significant (P<0.05) variation between the values in each column. ^{A,B} blue capital letters refer to a significant (P<0.05) variation between the values in each row.

The results of ROC curves analysis for the relative expression values of ISG15 in ewes on days 0, 6, 15 and 25 of pregnancy were presented in table 4. The value of the area under the curves (AUC) on both 0 and 6 days of pregnancy was 0.405, while the AUC on days 15 and 25 of pregnancy were 0.952 and 1.00, respectively.

Table 4: Results of the ROC curves for the data of relative expression values of ISG15 in ewes on days 0, 6, 15 and 25 of pregnancy

ROC curves values	Days of pregnancy			
	0	6	15	25
Area under curves (AUC)	0.405	0.405	0.952	1.00
Youden index cut-off value	0.065	0.504	0.679	0.291

Only on days 15 and 25 of pregnancy the AUC values were exceed 0.5, therefore the values of ISG15 expression at these two time points were used for the prediction of early pregnancy. Figure 2 presents the ROC curves for relative expression values of ISG15 in ewes on days 15 and 25 of pregnancy. On day 15 of gestation, the AUC was 0.952 ± 0.069. (with a 95% confidence interval for the area being between 0.817 and 1.087). The cut-off value of 0.679 was discriminated between pregnant and non-pregnant ewes. According to this cut-off value, six ewes were diagnosed pregnant and four ewes were diagnosed non-pregnant. The area under the ROC curves for relative expression values of ISG15 on day 25 of gestation was 1.00 ± 0.00 (with a 95% confidence interval for the area being between 1.00 and 1.00) The cut-off value of the relative expression value of ISG15 between the pregnant and non-pregnant ewes was 0.291. According to this cut-off value, seven ewes were diagnosed pregnant and three ewes were diagnosed non-pregnant.

Table 5 summarizes the accuracy, specificity, sensitivity, positive predictive value, and negative predictive value of ISG15 relative expression based on pregnancy outcomes confirmed by transabdominal ultrasonography on day 40 of gestation. On day 15 of pregnancy, six ewes were correctly diagnosed as pregnant, none were falsely diagnosed as pregnant, three were correctly diagnosed as non-pregnant, and only one was incorrectly diagnosed as non-pregnant. On day 25, seven ewes were correctly diagnosed as pregnant, none were falsely diagnosed as pregnant, three were correctly diagnosed as non-pregnant, and none were incorrectly diagnosed as non-pregnant. Consequently, the accuracy, specificity, sensitivity, and positive and negative predictive values of ISG15 on days 15 and 25 were 90%, 85.7%, 100%, 100%, and 75%, and 100%, 100%, 100%, 100%, and 100%, respectively (Table 5).

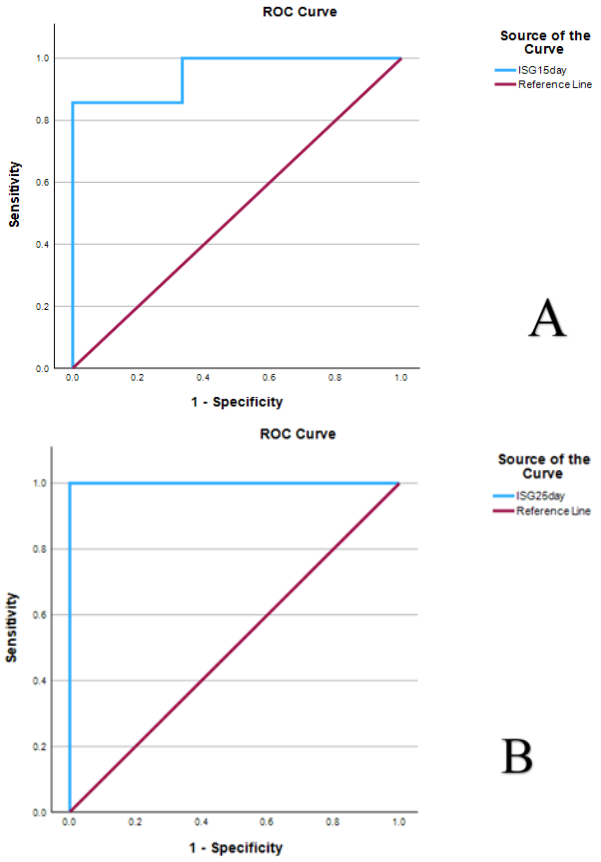

Figure 2: The ROC curves for the relative expression values of ISG15 on days 15 (A) and 25 (B) of pregnancy in ewes.

Table 5: Predictive results of the relative expression values of ISG15 for early diagnosis of pregnancy in ewes on days 15 and 25 of pregnancy

Values	Day of pregnancy	
	15	25
Number of ewes	10	10
diagnosed correct pregnant [(a)]	6	7
diagnosed incorrect pregnant [(b)]	0	0
diagnosed correct non-pregnant [(c)]	3	3
diagnosed incorrect non-pregnant [(d)]	1	0
Accuracy $^{(a+c)/(a+b+c+d)*100}$ (%)	90	100 *
Sensitivity $^{a/(a+d)*100}$ (%)	85.7	100 *
Specificity $^{c/(b+c)*100}$ (%)	100	100
Positive predictive value $^{a/(a+b)*100}$ (%)	100	100
Negative predictive value $^{c/(c+d)*100}$ (%)	75	100 *

* There was a significant ($P<0.05$) variation between the values in each row.

The accuracy, sensitivity and negative predictive value of the relative expression values of ISG15 were higher significantly ($P<0.05$) in ewes on day 25 of pregnancy in comparison to the values which were observed on day 15 of pregnancy. No significant variation was recorded in specificity and positive predictive value of the relative expression values of ISG15 in ewes which were calculated on days 15 and 25 of pregnancy.

Discussion

Transabdominal ultrasonography was employed in this study as a standard method consistent with numerous published reports (28,33-39) to facilitate the comparison and evaluation of ISG15 expression for early pregnancy diagnosis in Awassi ewes.

Several investigations have examined ISG15 expression for early pregnancy prediction in both large and small ruminants (13-19). In ewes specifically, ISG15 expression has been assessed in various organs and tissues to confirm pregnancy (21–27). To our knowledge, only a few studies have analyzed blood samples to evaluate the efficiency of ISG15 expression as an early pregnancy diagnostic marker in different sheep breeds (12,21,40,41), and no prior work has explored its use in Awassi ewes.

In the present study, ISG15 expression increased significantly on day 15 in pregnant ewes, corroborating the notion that an embryo secretes high levels of IFN-tau, which induces elevated ISG15 expression in the endometrium (14). Moreover, IFN-tau released from the uterus into the uterine vein can stimulate ISG15 expression in extra-uterine tissues, such as peripheral blood cells (42). These findings align with previous work that reported a similar modulation of ISG15 in the blood cells of pregnant ewes (21), as well as in the thymus (43), lymph nodes (24), bone marrow (22), endometrium (40), corpus luteum (13), and thyroid (27) of pregnant ewes.

Although ewes carrying twins exhibited higher ISG15 expression values than those with single pregnancies (2.034 ± 0.415 vs. 1.693 ± 0.468), this difference was not statistically significant. To our knowledge, no other studies have compared ISG15 expression values in ewes carrying twins versus single fetuses. However, Carvalho *et al.* (44) reported similar results in cows, they explaining that the differences in ISG15 expression between the single and twin pregnancies in their experiment were due to the number of embryos present during maternal recognition of pregnancy at the period between days 18 and 20 of pregnancy.

We used ROC curve analysis to determine the utility of ISG15 expression for early pregnancy detection in Awassi ewes. In our study, the AUC on days 0 and 6 of pregnancy were less than 0.5, while on days 15 and 25 of pregnancy were greater than 0.5. For any procedure that uses as a diagnostic marker, the AUC must be greater than 0.5 to be considered useful as a diagnostic test, and the AUC must be more than 0.8 to be considered as efficient diagnostic marker (45). For this reason, in this study the values of ISG15 expression on days 15 and 25 of pregnancy were used for early prediction of pregnancy in Awassi ewes.

Consistent with the relative expression value of ISG15 which were higher on day 15 than 25 of pregnancy, the calculated cut-off value of the ISG15 expression for discriminating between pregnant and non-pregnant ewes was higher on day 15 compared to 25 of pregnancy. Identical results to this finding have already been recorded for ISG15 expression in does as the cut-off value was >3.5 and >1.5 on days 15 and 25 of pregnancy, respectively (19). Based on the cut-off value of ISG15 expression on day 15 of pregnancy, only one ewe was incorrectly diagnosed as non-pregnant, while 6 of 7 ewes were correct pregnant diagnosed. But on day 25 of pregnancy, in spite of the values of ISG15 expression were lower than values that recorded on day 15 of pregnancy, all 7 ewes were correct pregnant diagnosed. These results are consistent with Kiyma *et al.* (41), who observed significant differences in ISG15 levels in 13 out of 16 ewes by day 15 but in all ewes by day 18 when compared to day zero of pregnancy.

The ISG15 expression test exhibited excellent accuracy on both days 15 and 25 (90% and 100%, respectively), primarily because only one of seven ewes was incorrectly diagnosed on day 15. Although other studies have also quantified ISG15 for early pregnancy detection in ewes, none to our knowledge have calculated the accuracy of this blood marker. Notably, the accuracy observed here exceeds that reported in several studies on cows: 83.3%, 85.6%, and 72.5% (15,16,46). Our results indicate that ISG15 expression more effectively detects non-pregnant rather than pregnant ewes, given the 100% specificity on days 15 and 25 (i.e., correct identification of all non-pregnant ewes). This finding

aligns with the biological premise that embryos secrete IFN-tau, which upregulates ISG15 in peripheral blood cells (42). Similar 100% specificity has also been documented on day 15 in does (19), whereas specificity values of 50%, 63.3%, and 74.5% were reported in cows on day 21 (15,16,46), and 80% in does on day 25 (19).

In contrast to this high specificity, the sensitivity of ISG15 expression was lower on day 15 (85.7%) because one pregnant ewe was misclassified as non-pregnant. By day 25, however, the sensitivity reached 100%, consistent with accurate classification of all pregnant ewes. This outcome may be attributable to the higher cut-off value adopted on day 15, which inadvertently led to one misdiagnosis. Comparable or closely matching sensitivity values for ISG15 expression have been reported on days 15 and 25 in does (90% and 100%, respectively) (19), as well as in cows on day 21 (90%, 96%, and 100% reported in three different studies) (15,16,46). Similar 100% specificity has also been documented by measurement of IFN-tau concentration in peripheral blood on days 15 and 25 of pregnancy in Awassi ewes (47).

Conclusion

We concluded that the ISG15 expression has a good accuracy which can be relied upon as an early pregnancy marker in Awassi ewes. The effectiveness of ISG15 expression for early prediction of pregnancy was more reliable on day 25 than 15 of pregnancy.

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Conflict of interest

The manuscript has no conflict of interest.

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كفاءة تعبير الجين المحفّز بالإنترفيرون ١٥ في الدم المحيطي للتنبؤ بالحمل المبكر في النعاج العواسي

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الخلاصة

تم تصميم الدراسة لتحديد كفاءة تعبير الجين المحفّز بالإنترفيرون ١٥ في الدم المحيطي للتنبؤ بالحمل المبكر في النعاج العواسي. تضمنت الدراسة عشر نعاج، ثلاث نعاج ذات حمل توأم، وأربع نعاج ذات حمل مفرد وثلاث نعاج غير حامل. تم جمع عينات الدم في الأيام ٠ و ٦ و ١٥ و ٢٥ من الحمل لتحديد القيم النسبية لتعبير الجين ISG15 باستخدام qRT PCR -. كانت نتائج قيم التعبير النسبي لـ ISG15 في النعاج الحوامل وغير الحوامل في الأيام ٠ و ٦ و ١٥ و ٢٥ من الحمل ٠,٠١٢±٠,٠٤٤ مقابل ٠,٠٠٩±٠,٠٤٩ و ٠,٠٨٣±٠,٢٠٣ مقابل ٠,٠٩٣±٠,٢٠١ و ٠,٥٩٤±١,٨٣٩ مقابل ٠,١٣٦±٠,٢٩٢ و ٠,٠٦٧±٠,٥٨٣ مقابل ٠,٠٥١±٠,٢٠٠ على التوالي. في كلا النعاج الحامل بتوأم ومفرد، زادت قيم التعبير النسبي لـ ISG15 بشكل كبير ($P<0.05$) في اليوم ١٥ من الحمل، وبعد ذلك انخفضت القيم بشكل كبير ($P<0.05$) في اليوم ٢٥ من الحمل، لم يتم تسجيل فرق كبير بين الحمل التوأم والمفرد. أظهرت نتائج منحنيات ROC أن المساحة تحت المنحنى في اليومين ٠ و ٦ من الحمل كانت ٠,٤٠٥، في حين أن المساحة تحت المنحنى في اليومين ١٥ و ٢٥ من الحمل كانت ٠,٩٥٢ و ١,٠٠، على التوالي. كانت قيم الدقة والنوعية والحساسية والتنبؤ الإيجابي والسلبي للتعبير النسبي ISG -15 في اليومين ١٥ و ٢٥ من الحمل ٩٠ و ١٠٠ و ٨٥,٧ و ١٠٠ و ٧٥ ٪ و ١٠٠ و ١٠٠ و ١٠٠ و ١٠٠ و ١٠٠ ٪ على التوالي. استنتج من الدراسة أن تعبير ISG15 له دقة جيدة يمكن الاعتماد عليها كعلامة حمل مبكرة في النعاج العواسي، وكانت فعالية تعبير ISG15 للتنبؤ بالحمل المبكر أكثر موثوقية في اليوم ٢٥ من اليوم ١٥ من الحمل.