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Assessing ISG15 Expression in Peripheral Blood Leukocytes During Early Pregnancy: A Potential Marker for Embryo Implantation and Early Pregnancy in Iraqi Does

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Abstract

THE article aimed to assess the mRNA-expression levels of Interferon-Stimulated Gene 15 (ISG15) in does, both pregnant and non-pregnant, during the early pregnancy period. Additionally, to determine pregnancy detection parameters using the ISG15 mRNA expression pattern. In this study conducted in Al-Saqlawiyah, Al-Anbar governorate, Iraq, 25 does and 5 bucks were utilized from March to October. The estrus induction protocol involved the use of intravaginal sponges containing Medroxy Progesterone Acetate for 12 days along with 500 IU PMSG at the time of sponge withdrawal. Estrus was identified, and the breeding bucks facilitated mating. Pregnancy status was assessed through transabdominal ultrasonography. Blood samples were obtained from does before estrus induction and after mating (on days 15 and 25 post-mating). Subsequently, RNA samples were extracted from peripheral blood leukocytes (PBLs), and ISG15 expression levels were evaluated using Real-Time PCR. The Receiver Operating Characteristic (ROC) curves were used to determine the efficiency of the ISG15 method in pregnancy detection. The results indicated a significantly higher ($P \leq 0.01$) expression of ISG15 in pregnant does on both days 15 and 25 compared to the control (before mating) and non-pregnant does during the same periods. Additionally, there were no significant differences observed between the two time frames within each non-pregnant and pregnant group. The ROC curves derived from the RT-PCR data on day 15 demonstrated a higher Sensitivity (Se) and Specificity (Sp) of 90% and 100%, respectively, with a dependable cutoff value >3.15. Moreover, on day 25, the ROC curves recorded 100% Se and 80% Sp at the designated cutoff point of >1.5. In summary, ISG15 exhibited a significant upregulation exclusively in pregnant does, while displaying only slight changes in mated/non-pregnant does. Due to its high accuracy in predicting pregnancy, it can serve as a reliable method for detecting implantation and early pregnancy in does.

Keywords: *ISG15*, RT-PCR, Early Pregnancy, implantation, Sensitivity, Specificity, Does.

Introduction

The Iraqi native goat is distributed all over the country, which is important for meat and milk [1]. It is characterized by high reproductive performance values such as high fertility rate, prolificacy, short puberty and litter size [2,3].

In Does, embryo implantation is crucial to pregnancy success, the successful embryo implantation process requires the co-regulation of multiple hormones and molecules to the formation of endometrial receptivity and control of embryo implantation [4]. The embryo reaches blastocyst satge at day 7 in culture in goats [5]. The Caprine embryo in the morula stage can be recovered from the uterus during flushing by day 6-7 after breeding [6]. Uninucleate and binucleate trophoblast cells represent a prevalent characteristic in ruminant placentae, constituting 15-20% of the trophectoderm, and they are the source of Interferon Tau (INF-τ) expression [7].

Interferon Tau plays a vital role in maternal recognition and preventing embryo rejection by inhibiting the immune modulation and response toward a newly formed conceptus [8-10]. Additionally, INF-τ blocks of the endometrial luteolytic mechanism and maintaining CL function during pregnancy [11,12].

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Additionally, INF-τ elicits upregulation of vast interferon-stimulated genes (ISGs) in the maternal tissues especially uterus [13], ovarian (luteal tissue) and extraovarian tissues like; blood cells, liver, spleen, lymph nodes and thymus throughout earlier gestation [14-17]. These ISGs; Transducer and Activator of Transcription (STAT) 1and STAT2, Receptor Transporter Protein 4 (RTP4), Oligoadenylate Synthetase 1 (OAS1), ISG15, Interferon-Induced Myxovirus Resistance Protein 1 (Mx1) and 2 (Mx2), Interferon Regulatory Factor (IRF) 1 and 9 that expressed in different maternal tissues and many cell types with vast effects [15, 16, 18].

The evaluation of mRNA upregulation of ISGs in PBLs is a candidate for a reliable peripheral detection marker of early pregnancy in ruminants because it is specific, reliable, and accurate for pregnancy diagnosis and it can be applied as a bio-marker for pregnancy case through the peri-implantation phase in embryo transfer and embryo loss to promote earlier rebreeding of cows [19-21] , it's better than other methods like P4 assay and ultrasonography for pregnancy diagnosis in earlier pregnancy period [22, 23].

The Does showed a high percent of early embryonic mortality (19%) between days 20-23, and the serum progesterone does show the non-significant difference between normal pregnant goats and goats that experienced embryonic death between days 7-20 post-conception, also ultrasound was not efficient for early embryonic losses detection at this period [24-26]. According to Okorie-Kanu *et al.* [27] study, economic losses happened because majority of slaughtered doe were pregnant. Therefore, pregnancy diagnosis is very crucial to minimize these losses and improve the efficiency of embryo transplantation by aiding to early pregnancy detection according to mRNA expression of ISG15. The study was designed to assess the mRNA expression levels of ISG15 in control, non-pregnant, and pregnant does during the early pregnancy period. Additionally, to identify the successful pregnancies and embryonic death based on the ISG15 mRNA expression pattern.

Material and Methods

Ethical approval

The experiment was carried out under the conditions of Ethics Committee, Faculty of Veterinary Medicine, Alfallujah University.

Experimental Animals

Twenty-five multiparous Iraqi does, age ranging between 2-3 years were studied along with five Bucks used for heat detection and breeding. The experiment was conducted at Al-Saqlawiyah, Al-Anbar governorate, Iraq. The experiment extended from March to October 2023. All experimental does were double-checked by ultrasonography (Chison ECO2/China), and it was confirmed that they were not pregnant.

The estrus was synchronized via using Medroxy progesterone acetate (Intravaginal sponge) for 12 days+ PMSG 500 IU at the time of progesterone withdrawal according to Kuru *et al.* [28].The onset of estrus was detected by observing the natural mating after two days post-PMSG injection. Every doe was separated after conceive by Buck. The blood was collected from all does (n=25) before synchronization, on days 15 and 25 post conceive (Day 0 = day of mating). All post-breeding does were examined by ultrasonography on days 60 to confirm the pregnancy status.

Quantitative Real-Time PCR for Determining ISG15 Expression

The collected for analysis of ISG15 gene expression (0.5 ml) evacuated into a collection tube containing 0.5 ml of TRIzol reagent (Thermo Scientific, USA) (1:1 ratio), then the qRT- PCR samples were frozen immediately freeze at -20^0C, and transported to the laboratory for qRT-PCR assay.

The total RNA of each sample was isolated from PBLs depending to Rio *et al.* [29], the RNA quantity was assayed by QuantiFluor® RNA System (Promega, USA) according to manufacturer's instruction.

Two pairs of primers (Macrogen/ South Korea) were designed to amplify the mRNA of the Caprine ISG15 and the housekeeping gene Beta-actin (housekeeping gene) (ACTB);Table 1).

TABLE 1. The ISG15 and housekeeping Primers used for qRT-PCR

Gene description	Accession No.	Sequence (5' → 3')	Amplicon size	Reference
Beta-actin (housekeeping gene) *(ACTB)*	DQ661647.1	Forward: TCGGCAATGAGCGGTTCC Reverse: ACYGTGTTGGCGTAGAGGTC	46	Chandrakar *et al*. [30]
Interferon-Stimulated Gene 15 *(ISG15)*	XM_027968447.3	Forward: GACCTGACGGTGAAGATGCT Reverse: TGATCTTCTGGGCGATGAAC	100	Mauffré *et al*. [31]

The RT-qPCR (MicPCR; Labgene Scientific/ Switzerland) was used to amplify the purified RNA. The reaction consisted from: ISG15 and ACTB gene, qPCR master mix (5 uL), MgCl2 (0.25 uL), reverse transcriptase (0.25 uL), 0.5 uL for each forward and reverse primer, nuclease free water (2.5 uL), and RNA templet (1 uL), the final volume was 10 uL.

The target and housekeeping genes were amplified in one-step, each sample is added to two PCR tubes (Two reactions). One tube is added as a negative control (Did not contain RNA sample).

The reaction lasted 90 min for each reaction, and the software program Mic PCR v.2 10.0 is used to manage the reaction conditions (Table 2).

TABLE 2. The reaction conditions for ISG15 and ACTB genes

Gene	RT-PCR cycling				
	Hold Steps		Cycling		
	Reverse transcription	Initial denaturation	Denaturation	Annealing	Extention
ISG15	37 °C for 15 min	95 °C for 5 min	95 °C for 20 sec	60 °C for 20 sec	72 °C for 20 sec
ACTB	37 °C for 15 min	95 °C for 5 min	95 °C for 20 sec	60 °C for 20 sec	72 °C for 20 sec
No of cycle	1 cycle	1 cycle	40 cycle		

The mean threshold cycle of ISG15 values (CT) was determined according to the formula of Livak and Schmittgen [32]:
ΔCt (control)=CT (gene)-CT(HKG)
ΔCt (patient or treated)=CT (gene)-CT(HKG)
ΔΔCt=ΔCt (patient or treated)- ΔCt (control)
Fold change =2 -ΔΔCt Normalized expression ratio

Statistical analysis

The data analysis was conducted via SAS (2018; v9.6) [33]. The ROC curves also utilized to find out the effectiveness of ISG15 by determined the senstivity (Se) and specificity (Sp) in does by using MedCalc statistical software (2016; v16.4) [34]. The AUC and Youden index (Yd) also used for pregnancy determined, Youden index = (Sensitivity + Specificity - 1).

Results and Discussion

The pattern of ISG15 mRNA expression in PBLs of early pregnant ewes

Relative abundance levels of *ISG15* mRNAs, in caprine PBLs of pregnant group compared with non-pregnant control groups, are illustrated in Fig. 2. Before induce estrus, the expression level of *ISG15* mRNA in basal level. Then, the expression increased significantly (P≤0.01) in pregnant does compare to control (before mating) and non-pregnant does in the same periods, same finding was also detected on day 25 PM. The mRNA of *ISG15* expression recorded a significant increase (P≤0.01) in non-pregnant does compared to control on days 15 and 25 PM, but the upregulation level was very low in both groups. Additionally, non-significant declined was detected in day 25 compare to day 15 PM (Table 3; Figs. 1&2).

TABLE 3.The ratio of *ISG15* fold changes for Pre-treatment, Inseminated/pregnant, and Inseminated/non-pregnant does

Group	Mean ± SD of *ISG15* gene expression				LSD value (P-value)
		Post-Treatment	Day 15	Day 25	
Pre-Treatment (Control)	A 1.11±0.43 ^{b}	**Pregnant**	10.42 ±6.48 ^{Aa}	7.71 ±2.21 ^{Aa}	4.954 ** (0.0002)
Non-Pregnant		**Non-Pregnant**	2.05 ±0.91 ^{Ba}	1.40 ±0.21 ^{Ba}	1.337 NS (0.652)
LSD value (P-value)	---		5.491 ** (0.00944)	3.668 ** (0.00261)	---

** (P≤0.01).

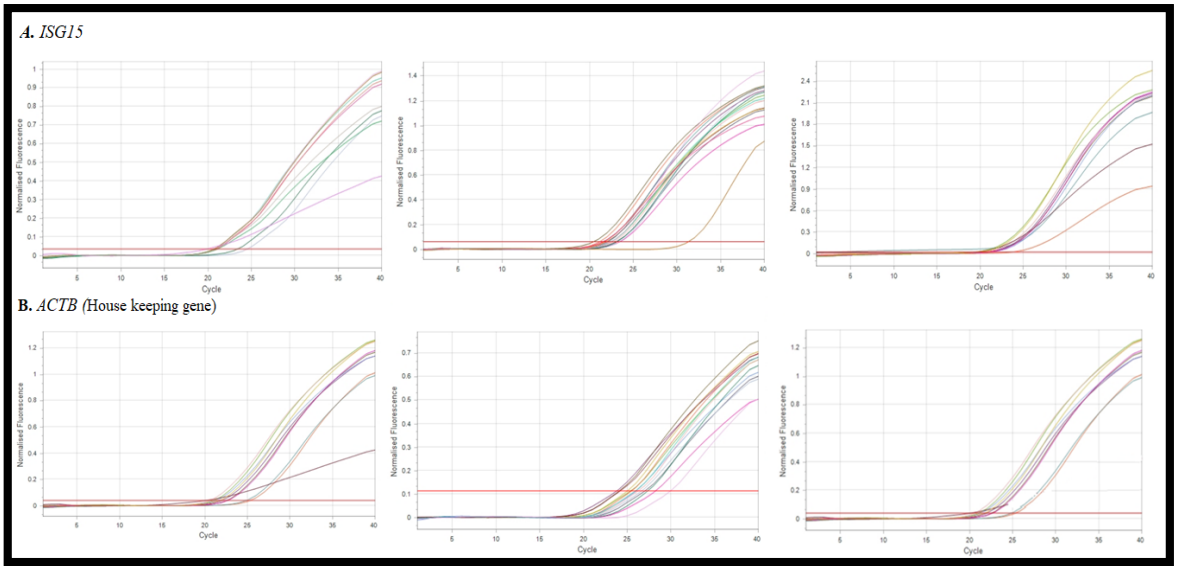

Fig. 1. The ISG15 mRNA gene expression for control, pregnant and non-pregnant does with the housekeeping gene

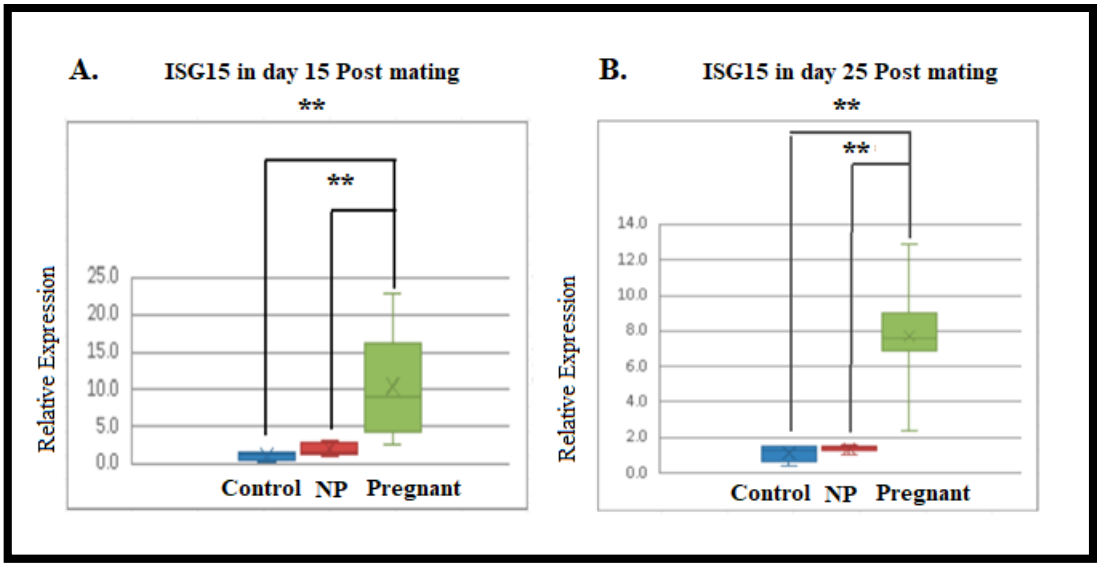

Fig.2. Ratio of ISG15 fold changes for control (blue bar), Inseminated/pregnant (green bar), and Inseminated/non-pregnant ewes (red bar) in day 15 (A) and 25 (B).

As demonstrated in Fig. 2, ISG15 abundance in the pregnant group was 5-fold greater than non-pregnant does on days 15 and 25 PM. Also is much higher than in the pre-treatment group. On day 25, the expression attenuated in PBLs collected on day 25 PM, but still elevated than both non-pregnant and control group.

These findings were agreed with Al-Samawi et al. (2021) [35] results, which find that relative expression of ISG15 mRNA was highly significant increased ($P<0.01$) in pregnant Does on days 15, 23, 25 and 35 PM, then declined on day 60 PM, there was non-significant difference between pregnant and non-pregnant.

The conceptuses of sheep expressed the Ovine TP-1 (INF-τ) during earlier gestation, it is responsible for pregnancy recognition between Days 15-17 [36]. In does, caprine TP-1 (INF-τ) was detected in serum on day 17 of pregnancy [37]. The Caprine cloned trophoblast cell line (uninucleate and the binucleate cells of trophoblast) expressed both mRNA and protein of INF-τ [7]. The term ISGs came due to the mRNA expression of several genes

influenced by INF-τ secretion at an early stage of pregnancy and were up-regulated in different maternal tissues in endometrial tissue; Both caprine STAT1 and STAT3 mRNA (apart from ISGs) increased in the endometrium on day 18 of pregnancy compared to day 5th and day 15th of gestation [38]. The same study also detected that protein levels of STAT1 and STAT3 increased significantly in endometrium within 24 h of INF-τ treatment. Additionally, the mRNA of ISGs is also expressed in extra uterine tissues such as CL, lymph nodes, thymus, liver, and peripheral leukocytes [14-17], that reflects a positive correlation between INF-τ concentrations and ISGs abundance. Gray *et al.* [39] detected those 180 endometrial genes (particularly; STAT1 and CXCL10) arose in response to pregnancy and infusion of INF-τ and P4. Although, among the expression of ISGs mRNA in various maternal tissues, the most important of them is the expression in PBLs. Because it is considered a candidate for reliable peripheral detection biomarkers of early pregnancy.

Numerous studies have indicated that certain ISGs are expressed in peripheral blood leukocytes (PBLs) during the early stages of pregnancy in ruminants: kose *et al.* [40] found that the mRNA expression of ISG15 increased between days 15 and 25 of PNM but decreased on day 21 after PGF2α injection on day 18 (embryonic death group). According to Mauffré *et al.* [31]; the mRNA expression of CXCL10, MX1, and STAT1 mRNA was significantly elevated in gravid ewes compared to non-gravid ewes, with respective fold increases of 8, 6, and 2.7 (P<0.001), but ISG15 recorded non-significant differences.

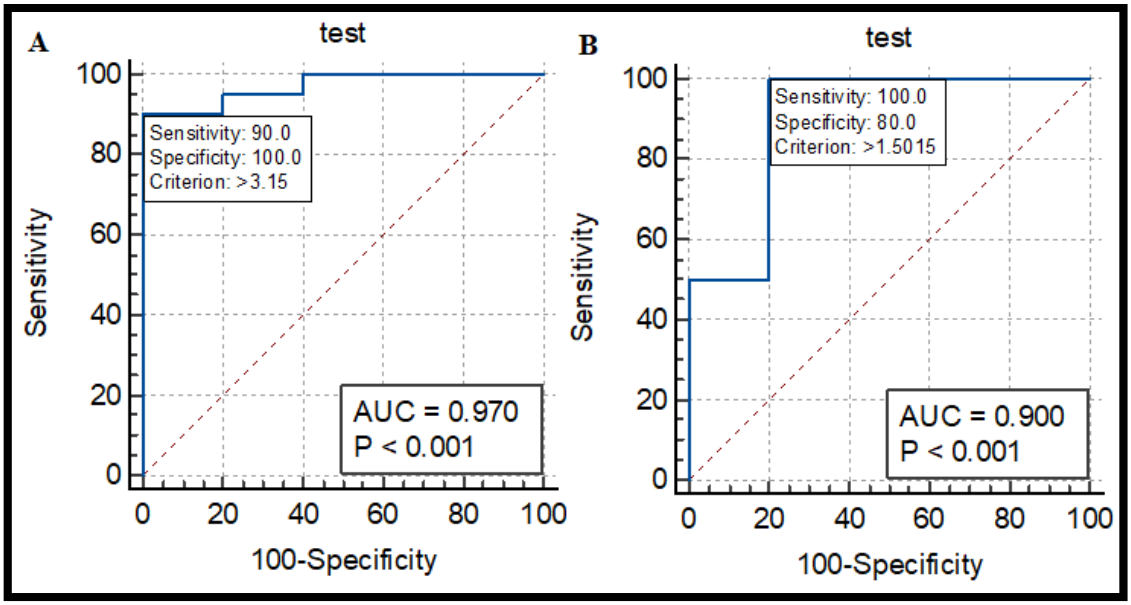

Fig.3. Receiver operating characteristic (ROC) curves for ISG15 ratio between gravid and non-gravid does

The ROC curves for the ISG15 ratio on day 15 exhibited a diagonal line across the center of the plot, indicating a suitable cut-off (>3.15). The Sensitivity and Specificity were 90% and 100%, respectively, with false negative and positive rates of 10% and 0%, respectively (Fig. 2A). The ROC curve for pregnant ewes on day 15 outperformed that of 25 PM. The AUC was 0.97, surpassing the lower limit of 0.70. On day 25 PM, the ROC curves for the ISG15 ratio (Fig. 2B) were deflected to the left but remained lower than those on day 15 (AUC 0.90) with a cut-off point >1.5. The sensitivity (Se) was 100%, surpassing the performance on day 15, while the specificity (Sp) was lower than on day 15 PM (80%), resulting in a 20% false positive rate.

These findings agreed with Mauffré *et al.* [31], who reported highly reliable outcomes for early pregnancy diagnosis using the expression of ISGs (STAT1, CXCL10, and MX1) on day 15 PI. The sensitivity was 100% for both STAT1 and CXCL10 and 88% for MX1, while the specificity was 100% for both CXCL10 and MX1 and 90% for STAT1. In cattle, achieving high true positive rates and low false positive rates for early pregnancy detection was possible using ISG15 in leukocytes at 19 to 20 PI, with sensitivity and AUC reaching 100% and 0.852, respectively [41].

Additionally, the outcomes of the present study corresponded with those of de Melo *et al.* [42], demonstrating that the sensitivity and specificity of ISG15 were very comparable to our findings on days 20 and 25 PM. The false negatives recorded on day 15 PM were attributed to the expression levels of 2 out of 20 samples falling below the cutoff point. Conversely, the false positive rate on day 25 was observed due to the presence of 1 sample out of 5 from non-pregnant ewes being lower than the cutoff point (>1.5). However, the higher false rate may be attributed to the small sample size.

This method of early pregnancy detection could be among the most effective due to its high sensitivity. It functions as an indirect yet specific predictor of pregnancy since the expression of ISG15 is influenced by the INF-τ signal, which is specific to embryos. Additionally, it proves reliable in determining non-pregnancy status during the early post-mating period due to failed conception or implantation (Sp), proving useful in cases where the CL fails to lyse at the anticipated time. Balaro *et al.* [43] documented instances of persistent CL in ewes linked to uterine pathology and embryonic death, hindering CL regression.

Conclusion

The study concluded that the expression of ISG15 was more pronounced in pregnant than non-pregnant does due to failed fertilization or early embryonic death during the 15-25 PM period. The ISG15 expression method demonstrated a very good Se and Sp rates, enabling the prediction of early pregnancy and pregnancy losses during this period utilizing blood samples collected under field conditions, as it relies on the presence of a viable embryo rather than an active corpus luteum.

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Conflict of interest

There are no conflicts of interest to be declared.

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تقييم التعبير الجيني ل ISG15 في كريات الدم البيضاء المحيطية أثناء الحمل المبكر: دلالة محتملة لانغراس الأجنة والحمل المبكر في الماعز العراقي

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الخلاصة

كان الهدف من الدراسة هو تقييم مستويات التعبير الحمض النووي الريبي المراسل للجين المحفز بالإنترفيرون 15(ISG15) في الماعز الحوامل وغير الحوامل أثناء فترة الحمل المبكر. بالإضافة إلى ذلك، لتحديد معايير الكشف عن الحمل باستخدام نمط التعبيرالجيني . ISG15 أجريت هذه الدراسة في الصقلاوية ، محافظة الأنبار، العراق، تم استخدام 25 من اناث الماعز و 5 ذكور من مارس إلى أكتوبر. يتضمن بروتوكول تحفيز الشبق استخدام إسفنجات مهبلية تحتوي على Medroxy Progesterone Acetate لمدة 12 يومًا مع 500 وحدة دولية من PMSG في وقت سحب الإسفنجة. تم التعرف على الشبق، وتم احداث التزاوج عن طريق التزاوج مع الذكور. تم تقييم حالة الحمل من خلال الموجات فوق الصوتية عبر البطن. تم الحصول على عينات الدم من قبل تحفيز الشبق وبعد التزاوج (في اليومين 15 و 25 بعد التزاوج). وفي وقت لاحق، تم استخراج عينات الحمض النووي الريبي (RNA) من كريات الدم البيضاء المحيطية(PBLs) ، وتم تقييم مستويات التعبير ISG15 باستخدام تفاعل انزيم البلمرة في الوقت الحقيقي. منحنيات الخاصية العملياتية للمستقبِل (ROC) المستخدمة لتحديد كفاءة طريقة ISG15 في اكتشاف الحمل. أشارت النتائج إلى ارتفاع معنوي (P<0.01)في تعبير ISG15 لدى الحوامل في كلا اليومين 15 و 25 مقارنة بمجموعة السيطرة (قبل التزاوج) وغير الحامل خلال نفس الفترات. بالإضافة إلى ذلك، لم تكن هناك فروق ذات دلالة إحصائية بين الإطارين الزمنيين داخل كل مجموعة حامل وغير حامل. أظهرت منحنيات ROC المستمدة من بيانات تفاعل انزيم البلمرة في الوقت الحقيقي في اليوم 15 حساسية أعلى (Se) ونوعية (Sp) بنسبة 90% و100%، على التوالي، مع نقطة قطع >3.15. علاوة على ذلك، في اليوم 25، سجلت منحنيات ROC حساسية 100% و نوعية 80% عند نقطة القطع المعينة >1.5. باختصار، أظهر ISG15 تضخيماً ملحوظًا بشكل حصري في اناث الماعز الحوامل، في حين لم يعرض سوى تغييرات طفيفة في اناث الماعز المتزاوجة/غير الحوامل. ونظرًا لدقته العالية في التنبؤ بالحمل، فإنه يمكن أن يكون بمثابة وسيلة موثوقة للكشف عن انغراس البويضة والحمل المبكر.

الكلمات المفتاحية: ISG15، RT-PCR، الحمل المبكر، الانغراس، الحساسية، الخصوصية، الماعز.