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Evaluation of the *STAT1* mRNA expression in peripheral blood cells as a predictive marker of early pregnancy in Awassi ewes

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Abstract

The primary objective of this study was to measure the expression profile of the signal transducer activator transcription 1 (STAT1) in the circulatory leukocytes of non-pregnant/pregnant ewes and test the possibility that it could be useful for detecting early pregnancy. Whole blood was collected from 20 ewes (control) and another 5 samples were taken on days (15, 25, 35, and 50). Also, blood was collected from 30 ewes on day 15 post insemination (PI) and another 10 samples were taken on days (25, 35, and 50) PI. Total RNA was isolated from peripheral blood leukocytes (PBLs), and *STAT1* expression levels were determined by quantitative real-time PCR (qRT-PCR). Gene expression for *STAT1* mRNA was 5.8-fold and 5.1 times higher in gravid as opposed to non-gravid ewes. The reliable cut-off point > 1.45 was established using the receiver operating characteristic (ROC) curve, and the accuracy (Acc) for detecting pregnancy was 82%, 93%, 70% and 60%, and the sensitivity (Se) was 83%, 80%, 60% and 33% and Specificity (Sp) 95% on days 15, 25, 35 and 50, respectively. The mRNA expression was lower in ewes that control non-pregnant than pregnant ewes on days 15 and 25. So The best period to detect pregnancy was 15-25 days PI.

Keywords: *STAT1*, mRNA expression, Pregnancy, ewes.

تقييم التعبير الجيني ل *STAT1* في خلايا الدم المحيطية كعلامة تنبؤية للحمل المبكر في النعاج ألعواسي

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الخلاصة

الهدف الأساسي من هذه الدراسة هو قياس نمط التعبير الجيني ل *STAT1* 1 (Signal transducer activator transcription 1) في كريات الدم البيضاء في الدورة الدموية للنعاج غير الحوامل / الحوامل واختبار إمكانية استعماله في الكشف عن الحمل. تم جمع الدم من 20 نعجة غير حامل (مجموعة سيطرة) وأخذت 5 عينات أخرى في الأيام (15, 25, 35, 50). كذلك تم جمع عينة دم من 30 نعجة في اليوم

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الخامس عشر بعد التسفيد (PI) وأخذت 10 عينات أخرى في الأيام (25 ، 35 ، 50). تم استخلاص الحمض النووي الرايبوسومي المرسل من كريات الدم البيضاء وتم تحديد مستويات التعبير عن *STAT1* بواسطة جهاز تفاعل انزيم البلمرة الكمي في الوقت الحقيقي (qRT-PCR). كان التعبير الجيني لـ *STAT1* أعلى بمقدار 5.8 ضعفًا و 5.1 مرة ($P < 0.05$) في النعاج الحوامل مقارنةً بغير الحوامل. تم تحديد نقطة القطع < 1.45 باستخدام منحنى الخاصية العملية للمستقبل (ROC) الذي أنشأ واعتمادًا على ذلك ، كانت الدقة (Acc) للكشف عن الحمل 82% ، 93% ، 70% و 60% ، والحساسية (Se) 83% ، 80% ، 60% و 33% في الأيام 15 ، 25 ، 35 و 50 على التوالي ، وسجلت النوعية % 95 (Sp) لجميع الفترات. كان تعبير الرنا المرسل أقل في نعاج السيطرة الغير حوامل مقارنة بالنعاج الحوامل في اليومين 15 و 25. لذلك كانت أفضل فترة للكشف عن الحمل هي 15-25 يومًا بعد التسفيد.

1. Introduction

There are lots of techniques for pregnancy diagnosis and early detection of embryonic death in ewes, including clinical, hormonal assays, immunologic detection, management methods, and, more recently, molecular detection. Moreover, the selection of it depends on the stage of gestation, the method's accuracy, and the tools available [1-4].

Interferon Tau (IFNT) or ovine trophoblast protein-1 oTP-1 is a pregnancy recognition signal in ruminants, secreted by trophoblast after embryo elongation on day 10 and ceases after day 21 [5-8]. In addition to its role in pregnancy recognition, IFNT has other functions such as anti-luteolytic and CL protective [9], preventing rejection and supporting progesterone secretion [10].

Several proteins, minerals, electrolytes, enzymes and blood biochemical profiles changed during gestation in sheep [11,12] and humans [13] along with changes in some genes expression [14] but it also several genes expressed in response to *IFNT*, which called Interferon tau Stimulated Genes (ISGs); it critical for conceptus expansion and attachment or implantation, it includes several genes: Interferon-myxovirusresistance 1 (Mx1)1 (Mx1) and 2 (Mx2), ubiquitin-like modifier (ISG15), STAT 1 and 2, radical S-adenosyl methionine-containing domain (RSAD2), oligoadenylate synthetase 1 (OAS1), receptor transporter protein 4 (RTP4) Interferon regulatory factor 1 (IRF1) and 9 (IRF9) in different maternal tissues and many cell types with vast effects [15-18].

Previous research found that some mRNA were expressed in specific maternal tissues during early pregnancy, such as *STAT1* and *Mx1* mRNA in ovine thymic tissue, which increased to their highest levels on days 16 and 25 of pregnancy, respectively, and may be involved in the coordination of the dam's immune response and tolerance at an earlier stage of gestation [18]. similarly in maternal liver [16], lymph nodes [17], and uterine endometrium [19].

Because the progesterone assay is considered non-specific for pregnancy prediction and detection [20], the ultrasonography detection Acc at an early stage of pregnancy is not optimal; it also needs experience and an instrument [21]. Therefore, the study was carried out to analyze the expression patterns of *STAT1* in peripheral blood using qRT-PCR at different periods of early gestation in pregnant and non-pregnant Iraqi Awassi ewes. Furthermore, using a blood sample, the Acc was determined as an alternative early gestational prediction in ewes.

2. Material and methods

2.1. Animal preparation and sample processing

Fifty multiparous Awassi ewes with an average age between 3-4 years were studied. All animals were housed in one flock. Four breeding rams were used for heat detection and breeding. The experiment was conducted at Veterinary College (Alfallujah University), latitude 33.37 and longitude 43.74. The experiment span extended from February to October 2022.

All experimental ewes were checked by 6.5 MHz transrectal and 4.5 MHz ultrasound (Chison ECO2/China), and it was confirmed that they were empty. then the animal groups were divided as follows:

Control group: Blood samples were taken once from 20 ewes and four different times (Days 15, 25, 35, and 50) from 5 ewes for qRT-PCR. All control ewes were periodically checked by ultrasonography (15, 25, 35, and 50) to ensure that they were not pregnant.

Post-synchronized group or post-mating group: Blood was collected from 30 ewes on day 15 due to the pattern of ISG expression around day 15 of gestation and at three different times (Days 25, 35, and 50) from 10 ewes for qRT-PCR. All post-mating ewes were examined by ultrasonography on days 35 and 50 to confirm that the ewes were pregnant.

The synchronization protocol; Progesterone (Intravaginal sponge) was inserted for 14 days+ PMSG 500 IU at time of progesterone withdrawal.

2.2. Blood collection and total RNA extraction, concentration measurement, and dilution

Blood was collected for analysis of *STAT1* gene expression for control and post-synchronized ewes by vena puncture (0.5 ml), then evacuated into a collection tube containing 400 μ l of TRIzol reagent (Thermo Scientific, USA); Blood- TRIzol should reach the final volume of 1 ml. The qRT- PCR samples were frozen immediately in a -20 degree freezer and transported to the ASCO center in Baghdad to be used in the qRT-PCR assay.

The total RNA of each sample was extracted manually from peripheral blood leucocytes according to Rio et al. [22]; The QuantiFluor® RNA System (Promega, USA) is specified to measure the quantity of RNA in each extracted RNA. According to the manufacturer's protocol, 200 μ L of diluted QuantiFluor dye was aspirated and added to a 0.5 ml PCR tube containing $> \mu$ L of RNA sample and left in a dark room for five min. The results were read by a Quantus Fluorometer. The ideal RNA concentration range is between 1-5 μ g; consequently, because of concentrations of RNA were high, the RNA samples were diluted by adding 20 μ L of ddH₂O to 2 μ L of RNA and centrifuged for 1 min. according to manufacturer instructions.

2.3. Quantitative real-time PCR of *STAT1* and housekeeping gene

Two pairs of primers (Macrogen/ South Korea) were designed to amplify the mRNA of the ovine *STAT1* and the housekeeping gene (Glyceraldehyde,3, phosphate dehydrogenase) (Table 1).

Table 1: The *STAT1* and housekeeping Primers used for qRT-PCR

Gene description	Symbol	Sequence (5' → 3')	Amplicon size (bp)
Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)	<i>GAPDH</i>	Mauffré et al. 2016 [23] F: GCTGACGCTCCCATGTTTGT R: TCATAAGTCCCTCCACGATGC	150
Signal transducer activator transcription 1	<i>STAT1</i>	Mauffré et al. 2016 [23] F: GTGGCGGAGAGTCTGCAGCA R: GGTGAGTTGGCATGCAGGGC	190

Total purified RNA (DNA-free) from gravid and non-gravid ewes was carried out by using RT-qPCR (MicPCR) (Labgene Scientific/ Switzerland). Regarding the *STAT1* amplify and the housekeeping gene (*GAPDH*), the qPCR Master Mix (5 ul), Reverse Transcriptase (0.25 ul), MgCl₂ (0.25 ul), Forward and Reverse Primer (0.5 ul + 0.5 ul), Nuclease Free Water (2.5 ul), and RNA (1 ul), sum of all components = 10 u.

During the reaction, cellular RNA was reverse-transcribed into cDNA, and then the target gene was amplified in one-step MicPCR (Labgene Scientific/ Switzerland). Each sample is added to two PCR tubes (two reactions), one for a target gene and another for a housekeeping gene. In the end, one tube is added as a negative control (containing the same mixture without RNA sample).

The reaction lasted 90 min, and the software program Mic PCR v.2 10.0 is used to manage the reaction conditions, the reaction conditions were set up as follows (Table 2).

Table2: The reaction conditions for *STAT1* and *GAPDH* genes

Gene	RT-PCR cycling				
	Hold Steps		Cycling		
	Reverse transcription	Initial denaturation	Denaturation	Annealing	Extention
<i>STAT1</i>	37 °C for 15 min	95 °C for 5 min	95 °C for 20 sec	63 °C for 20 sec	72 °C for 20 sec
<i>GAPDH</i>	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		

2.4. Statistical analysis

The analysis formula was utilized to determine the levels of gene expression according to Livak and Schmittgen [24]:

$$\Delta Ct (\text{control}) = Ct (\text{gene}) - Ct (\text{HKG})$$

$$\Delta Ct (\text{patient or treated}) = Ct (\text{gene}) - Ct (\text{HKG})$$

$$\Delta \Delta Ct = \Delta Ct (\text{patient or treated}) - \Delta Ct (\text{control})$$

$$\text{Fold change} = 2^{-\Delta \Delta Ct} \text{ Normalized expression ratio}$$

The data analysis was achieved via SAS (v9.6) [25]. The ROC curves were also utilized to find out the effectiveness of gene expression as a marker of pregnant ewes. The Least significant differences (LSD) and one-way ANOVA were done to estimate the significant variations among means. The AUC and Youden index (Yd) are also used for pregnancy determined tests based on candidate STATq values, Youden index = (Sensitivity + Specificity - 1).

The Kappa coefficient (measurement of agreement) was used to estimate the degree of agreement among tests (according to the area under the curve), and the analysis was presented by MedCalc Statistical Software [26].

The pairwise comparison was used to generally compare entities in pairs to judge which of each entity is preferable, it was used in ROC curves to test the statistical significance of the difference between the AUC two to six dependent ROC curves according to the Hanley and McNeil [27] method.

3. Results

3.1. The pattern of *STAT1* mRNA expression in PBLs of early pregnant ewes

The qRT-PCR assay revealed that *STAT1* mRNA was highly significant ($P \leq 0.001$) expressed in the PBLs of pregnant ewes on days 15 and 25 of pregnancy than on days 35 and 50, respectively, PI and also in the all periods of control. The *STAT1* gene is a part of ISGs that are prominently affected by IFNT, the findings showed that *STAT1* mRNA levels start up-regulation in PBLs of pregnant ewes on day 15, reach peaks between days 15-25 of gestation, then decrease on days 35, after which a further decline occurs on day 50 of pregnancy. The expression was low throughout the experimental periods (0, 15, 25, 35, and 50) and non-significant differences were recorded between the expression levels in the mentioned periods (Table 3) (Figure 1).

Table3: Ratio of *STAT1* fold changes for control and Inseminated/pregnant ewes in different periods.

Groups	STAT1 Gene expression by days (M±SD)					LSD
	0	15	25	35	50	
Control	1.35 ±0.48 b	1.261 ±0.20 b	1.415 ±1.06 b	1.002 ±0.27 b	0.989 ±0.33 b	1,176 NS
Ins./pregnant	-----	7.93 ±1.20 a	6.99 ±0.67 a	1.86 ±0.32 d	1.67 ±0.58 d	1.539 **

Means with a different letter are highly significantly different ($P \leq 0.01$).

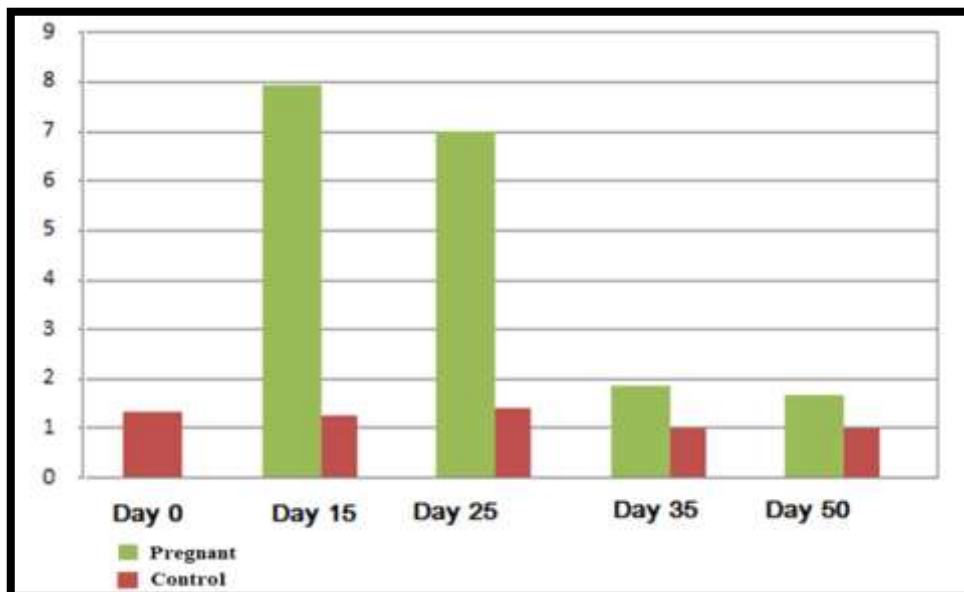


Figure 1: Ratio of *STAT1* fold changes for control and Inseminated/pregnant in different periods.

3.2. Pregnancy parameters according to the relative expression of *STAT1* expression in the ROC curve

The ROC curve determined the reliable cut-off point for *STAT1* mRNA expression as a continuous variable to determine the pregnant from the non-pregnant (control). At the cut-off point > 1.45 , the Se and Sp to identify pregnant and non-pregnant ewes were 83.3% and 95%, respectively (Figure 2 and 3).

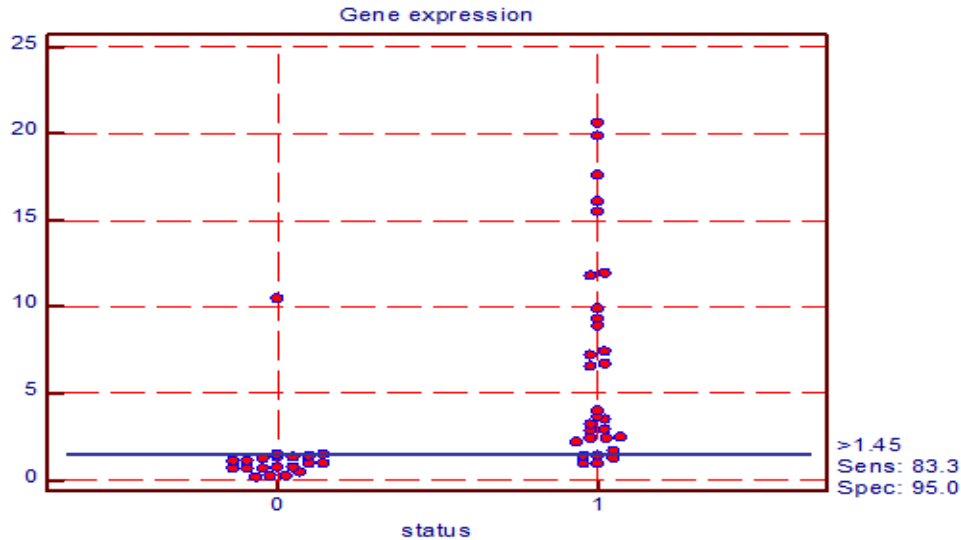


Figure 2: Distribution of ewes according to their relative expression for pregnant ewes on day 15 (1) and control ewes (0).

Using ultrasound as the gold standard for pregnancy detection on day 50, highly significant variations ($P < 0.0001$) were found in the levels of *STAT1* mRNA between the pregnant & non-pregnant ewes on days 15 and 25 post-conception. The ROC curves for *STAT1* showed a high aberration of the diagonal line to the left through the center on periods 15 and 25 PI (Figure 3a and b) and the AUC reaches 0.911 and 0.893 (above the lower limit of 0.70) with a cut-off > 1.45 at these periods. Additionally, higher Se of 83.3% and 80% and Sp of 95% were recorded on days 15 and 25 respectively. Therefore, the reliability of *STAT1* expression for early pregnancy diagnosis is very high. Furthermore, the Youden index was higher in these intervals, the values were far from 0 and closer to 1 (0.7833 and 0.7500 for days 15 and 25 respectively), which indicates that there were fewer proportions of false positives or false negatives; the results are summarized in Table 4.

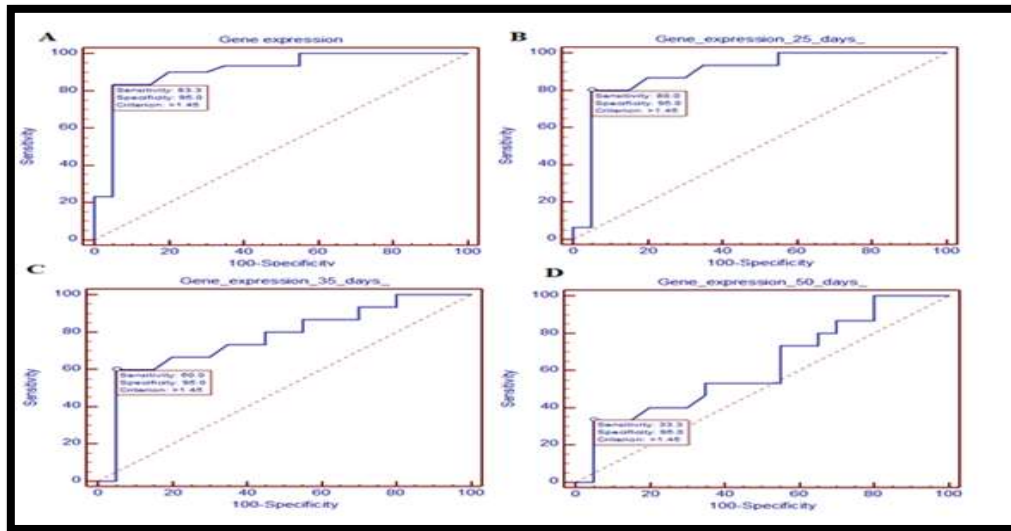


Figure 3: Receiver operator characteristic (ROC) curves for *STAT1* ratio between pregnant and non-pregnant on **A**. Represented the Se and Sp on day 15 with high deflection to the left, **B**. Represented the Se and Sp, with a moderate deflection on days 25, **C**. Represented the Se and Sp in days 15 with moderate deflection to the left in days 35, and **D**. Represented the Se and Sp on days 50 with low deflection to the left.

After day 25, a significant variations ($P > 0.01$) were detected between the two groups on day 35, while non-significant relationships ($P > 0.10$) were found on day 50 for *STAT1* mRNA levels. The ROC curves for ISG (*STAT1*) on days 35 and 50 presented no deviation through the center to the left of the line (Figure 3c and d) and both AUCs were around and below the acceptable minimum value (0.70), where 0.770 and 0.620 were recorded for days 35 and 50 respectively. Additionally, low Se (60% and 33.33%) and very low values for the Youden index (0.5500 and 0.2833) were found in the same periods due to the high ratios of false positives or false negatives (Table 4). The hierarchical classification did not differentiate between non-pregnant and pregnant ewes using *STAT1* gene expression on days 35 and 50 PI. Only 40% and 20% of pregnant ewes had a positive result according to the cut-off point >1.45 of gene expression on days 35 and 50, respectively (Table 4).

Table 4: The Statistical parameters for *STAT1* expression method in different pregnancy periods

Statistical scales	Day 15	Day 25	Day 35	Day 50
Area under the ROC curve (AUC)	0.911	0.893	0.770	0.620
Standard Error ^a	0.0455	0.0589	0.0845	0.0972
95% Confidence interval ^b	0.838 to 0.998	0.742 to 0.972	0.597 to 0.895	0.441 to 0.778
z statistic	9.032	6.677	3.197	1.234
Sensitivity	83.33	80.00	60.00	33.33
Specificity	95.00	95.00	95.00	95.00
Accuracy	0.82	0.93	0.7	0.76
Youden index	0.7833	0.7500	0.5500	0.2833
Associated criterion	>1.45	>1.45	>1.45	>1.45
Significance level P (Area=0.5)	<0.0001	<0.0001	0.0014	0.2171
SE a	0.0366	0.0589	0.0845	0.0972

^a Hanley & McNeil, 1983

^b Binomial exact

3.3. Compare the detection efficiency of *STAT1* expression between each two experimental periods

According to the Pairwise comparison of ROC curves in Table 5, there was non-significant relation of the AUC on day 15 when compared to day 25. Because of the high levels of AUC that were recorded on days 15 and 25, the non-significant differences mean it can detect pregnancy with the same efficiency on days 15 and 25.

In contrast, there is a significant rise was observed between the AUC of days 15 and 35 ($P \leq 0.01$), 15 and 50 ($P \leq 0.0001$), 25 and 35 ($P \leq 0.01$), 25 and 50 ($P \leq 0.001$), while, a non-significant relationship was noticed between AUC of days 35 and 50 give an indicator that pregnancy estimation capacity was batter on days 15 and 25 than other periods (Table 5).

Table 5: The comparison between the AUC of ROC curves for each pregnancy period

Gene_expression_15_days_ ~ Gene_expression_25_days	
Significance level	Non sig P = 0.1327
Gene_expression_15_days_ ~ Gene_expression_35_days	
Significance level	P = 0.0056
Gene_expression_15_days_ ~ Gene_expression_50_days	
Significance level	P = 0.0001
Gene_expression_25_days_ ~ Gene_expression_35_days	
Significance level	P = 0.0068
Gene_expression_25_days_ ~ Gene_expression_50_days	
Significance level	P = 0.0003
Gene_expression_35_days_ ~ Gene_expression_50_days	
Significance level	P = 0.0971

4. Discussion

4.1. The pattern of *STAT1* mRNA expression in PBLs of early pregnant ewes

The current study finds a significant difference in the mRNA *STAT1* gene expression in PBLs between non-pregnant and pregnant ewes in earlier stages of pregnancy, in the period that is confined between days 15 to 25. Subsequently, the upregulation of *STAT1* mRNA expression in gravid ewes decreased to a low level (on days 35-50) similar to the control level.

This finding was consistent with Mauffré et al. [23]; who observed that Gene expression for Signal transducer and activator of transcription 1 (*STAT1*) mRNA was 2.7-fold higher ($P < 0.001$) in pregnant compared with non-pregnant (controls) ewes on day 15 after estrus, 90% of non-gravid ewes had low *STAT1* mRNA level compared to gravid ewes. Furthermore, a significant variation in the abundance of the *MX1* gene mRNA was also observed in PLBs between pregnant and non-pregnant ewes [28].

The conceptuses of sheep expressed the mRNA of *IFNT* during earlier gestation between days 11 and 23 of gestation, with higher levels on day 13, the term ISGs came due to the mRNA expression of several genes influenced by *INFT* secretion at an early stage of

pregnancy and were up-regulated in different maternal tissues in uterine (endometrium) [29] and extra uterine tissues (CL, lymph nodes, thymus, liver and peripheral leukocytes [30, 16-18]). Gray et al. [31] detected that 180 endometrial genes arose (including the target gene in the current study) in responses to pregnancy, and infusion of, *IFNT* and progesterone particularly; *STAT1* and *CXCL10*.

Although, among the expression of ISGs mRNA in various maternal tissues, the most important of them is the expression in peripheral blood leukocytes. Because it is considered a candidate for reliable peripheral detection markers of early pregnancy, it shows promise for the early detection of pregnancy. The ISGs ratio started to rise from 19- 20 days in PBLs of pregnant cows in response to *IFNT*, while it remained unaltered in non-pregnant cows [32]. The ISGs included *ISG15*, *MX1*, *MX2*, and *OAS1* mRNA levels were significantly higher in PBL samples from pregnant compared to non-pregnant cows [33].

Because *INFT* ceases after day 21 of gestation, the *STAT1* abundance, as expected, decreased on days 35 and 50 PI. Nevertheless, ISG 15 gene expression declined on days 25, 30, and 45 PI in pregnant cows [34].

4.3. Pregnancy parameters according to the relative expression of *STAT1* expression in the ROC curve

Because there was no gold standard for diagnosing early pregnancy on days 15 and 25, we used ultrasound as a gold standard for pregnancy diagnosis on day 50, whereas the Acc of pregnancy assessment reaches 100% on day 40 PI [35]. Regarding the cut-off point > 1.45, higher Se and Sp were determined for pregnant ewes compared to the control. In this trial, *STAT1* appeared to be much more effective and accurate in detecting the empty ewes (Sp 95%) and pregnant ewes (Se 83.3%) with an Acc of 82% in the earlier stage of pregnancy on day 15 and (Se 80%, Sp 95% and Acc 93%) for day 25, with a highly significant difference ($P < 0.0001$) between the means of gene expression means of pregnant and empty ewes. The early pregnancy determining tests that depend on *STAT1* and *CXCL10* expression on day 15 PI in the Mauffré et al. [23] study revealed very reliable results, Se and Sp were 100% and 90%, respectively, higher than our findings. It may be because the RNA was extracted using a specific RNeasy Mini Kit, while our protocol depends on manual extraction, also the experiment was in specific environmental control conditions, while, the animals in the present study were reared on a farm. In cattle, one study pointed out that Se and AUC reached to 100% and 0.852, respectively for both *ISG15* and *Mx2* in leukocytes for early pregnancy detection at 19 to 20 PI, because high true positive rates and low false positive rates were shown at given cut-off point [36]. The Se did not reach 100% on days 15 and 25 PI, which may be because embryonic death can occur after day 15-25, that give false negative results. Diskin and Morris (2008) [37] mentioned that late embryonic and foetal death can occur after day 15 in sheep.

The higher Se and Sp for pregnancy assessment at an earlier stage of pregnancy could make it one of the best ways to early pregnancy detection. It is also considered an indirect but specific method for pregnancy prediction because *STAT1* expression is affected by the *INFT* signal (embryo-specific signal).

Transrectal ultrasonography was a very useful method for pregnancy prediction; however, according to Romano and Christians [38], the Se for pregnancy detection in ewes did not reach above 80% until days 17 and more, it recorded 0% and 27% on days 15 and 16, respectively, using 7.5 MHz transrectal ultrasonography. The Acc of the *STAT1* expression

method was better than that of ultrasound in the earlier stage of pregnancy on days 15 and 25. Additionally, the Acc is low before day 23 (50%), but increases by days 23- 30 (73 - 91%) [39], it is lower than our finding on day 25.

The early pregnancy diagnosis by Pregnancy Associated Glycoprotein (PAG) considers a pregnancy-specific method with very high Acc; a very high Acc (96%) for pregnancy prediction in ewes, but it began on day 18 and more [3].

Moreover, besides the progesterone assay non-specific for early pregnancy diagnosis, the Acc varied between the references at early pregnancy, it recorded 80% at period 15- 30 days in sheep [40] and 21 in doe [41], While Boscos et al. [42] study showed very high Acc (91.4%) for pregnancy prediction in ewes by day 17 PI (day 19 after sponge removal).

The very high Sp of the *STAT1* expression method (95%) gives superiority to this method on the progesterone assay. The Sp for the progesterone assay method was 65% [43]. However, the high value of this method in the diagnosis of pregnancy, the presence of a persistent CL in the absence of pregnancy may occur, resulting in an incorrect result (decrease the Sp).

This method is not specific and reliable to determine non-pregnancy status in the early period post-mating due to failure of CL to lysis at the expected time especially if uterine pathology and embryonic death occurrence that prevent CL regression [44].

On days 35 and 50 of pregnancy, low Se, Sp, and Acc were monitored compared to other methods (ultrasonography, progesterone, and PAG) [35, 39, 42, 43], therefore, it is less important than earlier pregnancy periods (15 and 25 PI). These results were approved by a pairwise comparison test of AUC in the ROC curves, it turns out that days 15 and 25 were highly significant in pregnancy detection compared to each days 35 and 50. The low AUC in the last two periods reflects the low expression in *STAT1* compared to the control and increases the false positive rates.

Conclusion

In summary, we have outlined that *STAT1* mRNA was up-regulated in pregnant ewes on days 15 and 25 PI, and declined after that (days 35 and 50), while low expressed in a non-pregnant group in different periods (0, 15, 25, 35 and 50). Taken together, these results suggest that the *STAT1* expression method was efficient for early pregnancy detection (15-25 days) in sheep, but not in subsequent periods because it is affected by the INFT pattern. The pairwise comparison between the AUC of the ROC curve revealed that the best period to detect pregnancy using this method was 15-25.

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Conflict of interest

There are no conflicts of interest to be declared .

Author contributions

Conceptualization, study design, sample collection, qRT-PCR and ultrasonography: Younis, Laith. Data analyses, Manuscript drafting, and Manuscript finalization: Hatif, Saad.

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