*Indian Journal of Animal Sciences* **87** (1): 49–52, January 2017/Article

# Early and efficient pregnancy diagnosis in ewes: expression profile of *ISG15* mRNA in peripheral blood leucocytes compared to transrectal ultrasonography

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Received: 16 June 2016; Accepted: 19 July 2016

ABSTRACT

The objective of this study was to compare the measurement of one of interferon-tau stimulated genes (ISGs; *ISG15*) expression in peripheral blood leukocytes (PBLs) with transrectal ultrasonography (TUSG) for early pregnancy detection in ewes. Ewes (46) were examined 18 and 25 days after mating by TUSG. PBL samples were collected from 16 pregnant ewes on the day of mating (d0) and on days 15 and 18 after mating. Total RNAs were extracted from PBLs and converted to cDNA. *ISG15* mRNA expression was detected using qPCR in triplicate. All pregnancies were confirmed by birth. In TUSG examinations on day 18, the embryonic vesicle and embryonic vesicle plus the embryo were visible for 14 and 32 ewes, respectively, of the 46 total. Both the embryonic vesicle and embryo were detected in 42 ewes on day 25. Four ewes out of 46 with a visible embryonic vesicle on day 18 were detected as non-pregnant on day 25. While significant differences in *ISG15* mRNA expression levels of PBLs were detected in 13 of 16 ewes on day 15, *ISG15* mRNA levels for all ewes were significantly different on day 18, compared with levels on the day of mating. The results suggested that profiles of *ISG15* mRNA can provide pregnancy detection earlier than TUSG.

**Key words:** Early pregnancy detection, Ewe, *ISG15* mRNA, Transrectal ultrasonography

Ewes are seasonally polyestrous animals, and a fertile ewe is desired to be pregnant as early as possible during the limited breeding season. Considering that intensive breeding systems and farms have a limited number of high-value stud rams, early detection of pregnancy is critical. Generally, mating and lambing records of many sheep flocks are omitted (Gursoy 2006). The collection of these records and detection of barren ewes is further complicated in sheep flocks where rams and ewes cohabitate. Therefore, early pregnancy detection may provide several important advantages in sheep flock management: (i) non-pregnant ewes can be quickly reinseminated; (ii) it facilitates culling or selling decisions; (iii) it decreases feeding and managing expenses by detecting infertile ewes in flocks; (iv) feeding, vaccination, weaning, and marketing can be programmed more effectively; and (v) it provides an opportunity for assisted reproductive techniques such as laparoscopic artificial insemination and embryo transfer in valuable commercial sheep flocks (Ishwar 1995).

Unfortunately, the development of early pregnancy diagnosis methods for the small ruminant industry receives a very restricted funding compared to the dairy industry, limiting the available methods. An ideal pregnancy diagnosis method should be non-invasive, cheap, reliable, practical, repeatable, and easily applied during early pregnancy (Ishwar 1995). However, none of the evaluated methods comprises of all of these properties at the same time so far. Currently, the preferred method in field conditions is real-time transrectal/transabdominal ultrasonography (USG). The main advantage of transrectal USG over transabdominal USG is earlier detection of pregnancy (Ishwar 1995). Observed in small echogenic areas, the accumulation of embryonic fluids in uterine lumen is the accepted first sign of pregnancy in ultrasonography. After this first sign, the reliability of the ultrasound based diagnosis reaches 100% with observation of the embryo and its heart beat in the next few days (20 days after mating: Romano and Christians 2008).

Expression profiling of interferon-tau-stimulated genes (*ISGs*) in peripheral blood leucocytes (PBLs) is a promising new technique for early pregnancy diagnosis in ewes (Ott *et al.* 2014). Yankey *et al.* (2001) showed that expression of MX1 and MX2 transcripts significantly increased in peripheral blood leukocytes in ewes. Similar results have been demonstrated in many studies of dairy cows (Oliveira and Hansen 2008, Kose *et al.* 2014). This study compared the efficacy of measuring *ISG15* expression in peripheral blood leukocytes with transrectal USG as a means of early

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pregnancy diagnosis in ewes. Among *ISGs*, increased expression *ISG15* was chosen for early pregnancy diagnosis based on the results of our previous study, which indicated strong expression profile of *ISG15* during early pregnancy in ewes (Kose *et al.* 2016).

## MATERIALS AND METHODS

*Animal material:* Healthy and fertile Awassi crossbred ewes (46), 2–5 year-old were kept on the pasture during the day and were placed in pens during the night. Ewes were fed diets formulated to meet the nutritional requirements for 3–5 year old, non-lactating sheep (59 kg mature body weight). Feed was withheld for 24 h before the transrectal USG examinations. Fresh water was available *ad lib.* The estrous cycles of ewes were synchronised by two intramuscular injections of 75 mcg d-cloprostenol at 10–day intervals. Ewes were mated by fertile rams on day zero (d0) after estrus detection by a teaser ram.

*Pregnancy diagnosis with transrectal ultrasonography*

Ewes (46) were examined by transrectal USG on days 18 (d18) and 25 (d25) after mating. Transrectal USG examination was done on a backward tilted table. During the examination, the back legs of the ewe were fixed in the vertical crowbars of the table. Faeces were removed from the rectum with a lubricated (carboxymethyl-cellulose) finger before the examination. A real-time ultrasound machine (high resolution, 7.5 MHz linear transrectal probe in probe holder) was operated by a skilled veterinarian. During the examinations, the lubricated ultrasonography probe was inserted into the rectum with slow circular rotations until the vesica urinaria or uterine horns were discernible. Uterine horns localised in the craniodorsal part of vesica urinaria were scanned slowly and carefully. Criteria including embryonic vesicle, embryonic vesicle plus embryo, and embryonic heartbeat were used for pregnancy diagnosis. All pregnancies were confirmed by birth.

*Pregnancy diagnosis with expression profile of ISG15 mRNA in peripheral blood leucocytes:* Expression profiles of *ISG15* mRNA in PBLs were examined in 16 ewes on d0, d15 and d18. The PBLs were isolated according to Kurar *et al.* (2011). Briefly, blood samples, collected into a 10-ml tube containing Na-EDTA, centrifuged at 300 × g for 20 min at 4°C. The buffy coat was harvested and resuspended in 1:5 v:v 0.87% Tris-$NH_4Cl$ lysis buffer. Samples were incubated at 37°C for 10 min and then centrifuged at 300 ´ g for 10 min. The PBL pellet was washed with 10-ml ice-cold PBS buffer and subjected to total RNA extraction. The RNA isolation, quality control, genomic DNA removal by DNase-I, and cDNA synthesis procedures were conducted following Atli *et al.* (2010). Briefly, total RNA isolation was performed using TRIzol reagent. RNA samples (2 µg) were cleaned of possible genomic DNA contamination by DNase-I treatment and were then subjected to reverse transcription to synthesise first-strand cDNA using the revertAid™ First-Strand cDNA Synthesis Kit, according to the manufacturer's instructions. Primers for *ISG15* were derived from known ovine sequences using Primer3 and the NCBI database (http://www.ncbi.nlm.nih. gov/). The gene expression profile of *ISG15* was evaluated on blood sampling days (d0, d15 and d18). The reaction was set up as follows: 5 µl SYBR Green Master Mix (2×), 5 pmol of each primer (Table 1), 1 µl cDNA, and $ddH_2O$ to a final volume of 10 µl. Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation, annealing, and amplification (95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec) on a Real-Time PCR System. Melting curve analysis was performed as follows: 95°C for 1 min, followed by fluorescence measurement at 1°C increments between 55°C and 95°C. A negative control with no cDNA template was included in each run. The entire procedure from RNA extraction to real-time PCR was performed twice as a technical replicate. Real-time PCR was performed in triplicate for each gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalisation of the real-time PCR data. We validated the consistency of steady-state GAPDH concentrations in the PBLs in preliminary experiments (unpublished results). Negative controls with no cDNA template and RT negative controls were included in each run. To verify reaction specificity, amplification products were evaluated after separation on a 2% agarose gel. To determine the dynamic range and amplification efficiencies of the real-time PCR assays for each gene product, amplifications were performed using specific primers in duplicate on a serial dilution of specific pooled cDNA. The amplification efficiency of qPCR was 95–105% for all genes. Normalised values with GAPDH for each individual ewe on day 15 and 18 of pregnancy were compared to its day zero value using the algorithm upon which the mathematical model is based. This basis depends on the PCR efficiency of each gene investigated and the mean deviation in Ct between groups. The expression ratios were considered statistically significant at $P<0.01$.

Table 1. Pregnancy examination results with transrectal ultrasonography on days of 18 and 25

| Day of pregnancy | Number of ewes with embryonic vesicle | Number of ewes with embryonic vesicle and embryo | Total |
|---|---|---|---|
| 18 | 14 | 32 | 46 |
| 25 | - | 42 | 42 |

Table 2. Significantly increased ISG15 mRNA expression in ewes due to pregnancy on days of 15 and 18

| Day of pregnancy | Number of significantly increased expression ISG15 mRNA in ewes |
|---|---|
| 15 | 13/16 (81.25%) |
| 18 | 16/16 (100%) |

## RESULTS AND DISCUSSIONS

Successful herd management in livestock is directly related to early and accurate pregnancy diagnosis. In this study, we studied and compared a molecular technique (measurement of interferon-tau stimulated gene expression in peripheral blood leukocytes) with transrectal USG in terms of early pregnancy detection in ewes.

*Transrectal USG:* The results from 46 ewes examined by transrectal USG are summarised in Table 1. On d18, the embryonic vesicle was detected in 14 ewes, while both embryonic vesicle and embryo were detected in 32 ewes. Seven days after that, both the embryonic vesicle and embryo were detected in 42 ewes. The remaining four ewes, which had only a visible embryonic vesicle on d18, were detected as nonpregnant on d25.

In sheep, one of the most widespread uses of USG is pregnancy diagnosis. Compared with transabdominal application, transrectal application allows for earlier pregnancy diagnosis, due to the shorter distance between the uterus and probe. Embryo viability, gender, and gestational age can also be detected with high accuracy using transrectal USG. There are several risks associated with this technique, however, including bleeding and perforation of rectal mucosa, which may increase the likelihood of infection. These risks can be reduced by preventive practices such as fasting ewes for 12 h before examination and restraining them during the examination (Karen *et al.* 2004, Romano and Christians 2008).

Several observable criteria are used for pregnancy diagnosis, including the embryo, embryonic heart rate, the placentoma, and the amniotic sac. We observe the embryonic sac and embryo on d18 as a sign of early pregnancy diagnosis. Additional reliable criteria for early pregnancy diagnosis, such as embryonic heartbeat and signs of embryonic viability, should be observed during the examination (Martinez *et al.* 1998). Although the heartbeat can be detected as early as the 18th or 19th day of pregnancy, heartbeat may not be observed in all pregnant sheep until as late as day 23 (Martinez *et al.* 1998). In this study, embryo plus embryonic vesicle were detected in 70% (32/46) of the ewes on day 18, while only embryonic vesicle was observed in 30% (14/16) of the ewes at this stage. Although the embryonic vesicle can be detected as early as day 12 of pregnancy, it is noted that diagnoses based solely on the embryonic vesicle have low accuracy and reliability (de Bulnes *et al.* 1998). Study was consistent with previous reports that the embryo can be first detected 16 days after pregnancy, and the detection rate increased up to 90% on day 18, and reached to 100% by day 20 (Romano and Christians 2008).

Before day 25, the rate of false positives using transrectal USG can be as high as 35% (Karen *et al.* 2004). Similar to cows, false positive pregnancy diagnosis in ewes can be caused by accumulations of fluids in the uterine lumen due to estrus, pyometra, mucometra, and extra uterine organs such as intestines (Karen *et al.* 2004). In our study, four ewes were considered to be pregnant by observation of the embryonic vesicle on d18, but were found not pregnant on d25. Embryonic death within the previous 7 days or fluid accumulation during the follicular phase of the estrous cycle could explain the false positives. Many studies have shown that embryonic losses occur in ruminants during maternal recognition of pregnancy within the first three weeks (Michels *et al.* 1998, Wilmut *et al.* 1986).

*Expression profile of ISG15:* While significant differences in *ISG15* mRNA expression levels of PBLs was detected in only 13 of 16 ewes on d15 compared to d0, significant levels were measured for all ewes on d18 (Table 2, $P<0.01$).

Previous studies were also indicated increased expression profiles of ISGs in PBLs in pregnant ewes on day 15 (Oliveira *et al.* 2008, Yankey *et al.* 2001). The current results support those findings and show that *ISG15* mRNA expression in PBLs in the majority of ewes (13/16) increased significantly by d15, before the next expected estrus. These results suggest that this molecular technique provides an advantage over transrectal USG, and offers an alternative, non-invasive method for early pregnancy detection. Considering that the length of one estrous cycle in ewes is around 17 days, earlier pregnancy detection allows reinsemination without missing the estrous cycle, which promotes greater economic gains in sheep flocks. In our study, three of 16 ewes failed to reach significant expression levels for *ISG15* mRNA on d15. Insufficient interferon-tau production due to delayed embryonic development or different ovulation time after mating may explain this, because bigger embryos produce more interferon-tau, which would induce more ISGs expression (Kose *et al.* 2016, Matsuyama *et al.* 2012).

In conclusion, earlier study that investigated *ISGs* including *ISG15, RTP4*, and *MX1*, the expression of *ISGs* was significantly increased in endometrium in the presence of an embryo on day 13 (pre-luteolytic stage of the estrous cycle) but not in PBLs (Kiyma *et al.* 2015). The first 15 days after mating is a critical period for early pregnancy diagnosis when using these methods, and future studies should focus on this time period. While our study suggested that profiles of *ISG15* mRNA provide a noninvasive alternative to transrectal ultrasonography for early pregnancy detection, the practical use and economic impact of this technique requires further investigation.

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