ORIGINAL ARTICLE

Unveiling the Role of *MX1* and *MX2* Gene Expression in Early Pregnancy of Kari Ewes: Insights into INF-tau Signaling and Potential as a Predictive Biomarker for Pregnancy Detection in Domestic Ruminants

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Abstract

Myxovirus resistance (MX) genes are relevant to a family of interferon (IFN)-stimulated genes. In domestic ruminants such as ewes and cattle, the *MX* genes have two isoforms called the *Myxovirus Resistance 1 (MX1)* gene and the *Myxovirus Resistance 2 (MX2)* gene. INF-tau (INFt) is released from embryonic trophoblast cells in the early days of pregnancy and induces expression of MX genes in the uterine endometrium. In the present study, we summarized the relative expression of the *MX1* and *MX2* genes during the early days of pregnancy in Kari ewes. Blood samples were taken from inseminated and non-inseminated (control) ewes on different days. Peripheral Blood Monocytes (PBMC) were isolated, and with the addition of *MX1* and *MX2* gene primers, complementary deoxyribonucleic acid (cDNA) was synthesized by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The cDNA was then amplified by rounds of PCR amplification. The amplified product was run on a 2% Agarose gel. The results showed expression of the *MX1* and *MX2* genes only in pregnant ewes, while no expression was observed in non-pregnant or control ewes. Expression in pregnant ewes was observed on days 14, 16, 17, and 18 after insemination. When comparing the *MX1* and *MX2* genes, the expression of the *MX2* gene was relatively higher than that of the *MX1* gene on all experimental days. The result suggested that INF-tau stimulated MX genes are expressed during the early days of pregnancy and are involved in the establishment of pregnancy. These results justify the development of this biomarker as a predictive tool for the early detection of pregnancy in ewes and other domestic ruminants.

Abbreviations

Myxovirus Resistance **(MX);** Peripheral Blood Monocytes (PBMC); Interferon tau (IFNt); Oligoadenylate Synthetase 1 (OAS1); Interferon-Stimulated Genes (ISGs); Interferon-stimulated gene 15 ubiquitin-like modifier (ISG15); Signal Transducers And Activators (STAT) 1; IFN regulatory factor 1 (IRF-1); Peripheral Blood Leukocytes (PBLs).

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Introduction

The early stages of blastocyst implantation are vital for the maintenance of pregnancy, as they comprise essential events. All through the peri-implantation phase, the developing blastocyst depends on the secretion of histotrophic substances within the uterine environment [1,2]. Comprising a mixture of nutrients, enzymes, hormones, growth factors, and transport proteins, this combination is under the regulation of communication between the embryo and the mother, known as embryo-maternal cross-talk [3,4]. Interferon tau (IFNt) is an important pregnancy-related signal to the maternal system. In the initial maternal recognition mechanism in ruminants, IFNt is one of the first molecules implicated [5]. In cattle, around days 14-15 of pregnancy, the trophectodermal cells of the blastocysts release this protein [6]; and days $16-25$ in buffalo [7]; days 13-17 in ewe [8]; and rises with the conceptus elongation [9-11]. This type of interferon controls immunological and luteotropic systems for effective implantation of embryos since it has antiviral, immunomodulatory, and antiproliferative capabilities [12]. Moreover, during the release of IFNt, it induces perceptible temporal changes in local and peripheral tissues [11-14]. In the uterus, IFNt inhibits luteolysis by preventing the release of prostaglandin, resulting in the maintenance of corpus luteum function [15]. In fact, IFNt activates the expression of Interferon-Stimulated Genes (ISGs), including $MX1$, $MX2$, interferon-stimulated gene 15 ubiquitin-like modifier (ISG15), and $2^{/},5^{/}$-oligoadenylate synthetase 1 (OAS1), in many cells, for instance luteal, endometrial, and peripheral blood cells [16,17]. In bovines, numerous studies have proved that ISG expression rises during early gestation in peripheral blood leukocytes [18-21]. In the endometrium, IFNs activate the expression of more than 750 other IFN-stimulated genes [22] but it is also possible that these genes are conferred by other type 1 interferons, such as interferon-stimulated gene 15 (ISG 15), and $MX\,1, MX\,2$ [22,23]. After infusion of IFN into the uterine vein, expression of ISG-15 was found in ovine corpus luteum cells [24]. The maintenance of a healthy corpus luteum is achieved through this interaction, which abolishes the luteolytic impulses of PGF2. Conceptualized in the endometrium, IFN tau acts locally to regulate many functions, including transcription Signal Transducers and Activators (STAT) 1 and 2, IFN regulatory factor 1 (IRF-1), and IFN regulatory factor 9 (IRF-9). are activated in the endometrium of pigs, dairy cows, ewe, ponies, humans, and mice [13]. $MX1$ and $MX2$ expression corresponds to high levels of progesterone 4 (P4) in the midst of the oestrous cycle, suggesting that P4 in uterus may also manage $MX1$ and $MX2$ gene expression [25]. The expression of ISGs like $MX1$ and $MX2$ gene was seen in peripheral blood monocytes (PBMC) of pregnant ewes, demonstrating contrast in gene expression among pregnant and non-pregnant ewes and cows [26]. In ewe's endometrium, IFN-tau stimulates $MX1$ and $MX2$ genes. The main function of $MX1$ and $MX2$ genes is to protect conception from viral diseases. $MX1$ and $MX2$ genes have the ability to counteract the luteolytic component to promote early pregnancy [27]. The MX genes is activated by type I IFN, which is a potent inhibitor of viral replication [28]. Actual MX genes expression is activated in all cells possessing type I IFN receptors and has been used as a marker for viral contamination [29].

Currently, there are several methods for detecting pregnancy in ewe, including visual observation, ultrasound, and hormonal assays. However, these methods are often costly, time-consuming. Recent research has shown that the expression of certain genes can be used to detect early pregnancy in ewe with greater accuracy and efficiency. Specifically, the $MX1$ and $MX2$ genes have been identified as potential biomarkers for early pregnancy detection in ewe. Thus, the objective of this study was to quantify interferon-stimulated genes ($MX1$ and $MX2$) in PBMCs during peri-implantation and early pregnancy in kari ewe. Additionally, the possible relationship between $MX1$ and $MX2$ mRNA expression was also investigated. Ultimately, the findings of this study could contribute to the development of a more cost-effective and reliable method for early pregnancy detection in ewe, with potential applications for other livestock industries as well.

Methodology

Animals and experimental design

The proposed study was carried out at the Institute of Biotechnology and Genetic Engineering (IBGE), University of Agriculture, Peshawar, Pakistan. The experiment was carried out according to the guidelines of the ethical committee of the University of Agriculture, Peshawar, Pakistan. In the current research work, a total of 6 ewes were selected including 1 ewe kept as a control. Animals selection and experimental design. Ewe were selected from the

Kari breed found in the Chitral region of Pakistan that had been put under synchronization. Regular clinical exams have been carried out, particularly before estrus synchronization, to rule out conditions such as endometritis, mastitis, and metabolic problems. Initial data for all animals were kept, and a protocol was adopted for bringing all the animals into the status of synchronized cyclicity. The following protocol was followed.

1. 0.4-0.6 ml of Cylomate®was injected into all screened ewe.

2. After 7 days, again, 0.4-0.6 ml of Cyclomate® was injected.

3. After 12 hours of cyclomate® injection, 1 ml of GnRH was injected.

4. 12 hours after the GnRH injection, rams were introduced to the flock.

Rams were introduced to the flocks for 24 hours and were closely observed to ensure proper mating. After mating, blood samples were collected from the jugular veins in a 10-ml EDTA tube. The blood samples were collected on day 8 of insemination, followed by samples on alternate days until day 13. From day 13 to day 18, blood samples were collected daily. All the EDTA tubes were placed in an ice box while being brought to the laboratory for further processing. The animals were grouped as pregnant, non-pregnant, and control.

Pregnancy diagnosis

Pregnancy was diagnosed through both transrectal ultrasonography and specific gene expression (*MX* genes). The first pregnancy was detected by gene expression within 18 days of mating. Later, it was confirmed through transrectal ultrasonography on day 30.

Isolation of PBMCs

The collected blood was centrifuged at 12000 rpm for 15 minutes to separate the three layers, and then kept on ice to visualize it clearly. Removed the upper layer and collected the middle, thin layer of PBMCs, which were then aspirated with great care. The collected PBMCs were then subjected to further processing. The isolated PBMCs were stored at -80°C (not 800 oc) until RNA extraction.

Total RNA isolation, reverse transcription, and PCR amplification

After PBMC isolation, total RNA were isolated using Trizol method. The concentration and purity of the extracted RNA were evaluated using the NanoDrop2000 equipment from Thermo Scientific (Waltham, MA, USA). The purity of RNA was measured by calculating the A260/280 ratio, which was recorded to be between 1.8 and 2.0. Furthermore, the RNA concentration in all samples was above 150 ng/µl. A first-strand cDNA synthesis kit (thermos-scientific catalog No. 1622) was used to obtain cDNA. Reverse transcriptase polymerase chain Reaction (RT-PCR) was used for cDNA synthesis. cDNA amplification was done through PCR by using **MX1, MX2, and GAPDH** gene primers (Table 1). Forward and reverse sequence of primers for all the genes were taken from the literature [14]. The total PCR amplification solution (20 µl) comprised 10 µl of master mix, 1 µl of cDNA, 1µl of primers (forward and reverse) (1+1 = 2 µl), and 7 µl of dH2O.The reaction procedure was as follows: predenaturation at 94°C for 5 min, denaturation at 94°C for 30s, annealing at 53°C for 30s, and extension at 72°C for 30s for 40 cycles. Thereafter, samples were stored at 4°C.

Gel electrophoresis

The PCR-amplified product was subjected to agarose gel electrophoresis, which involved preparing a gel in 1xTBE buffer with ethidium bromide as a fluorescent dye. The PCR-amplified product was then run on the 2% agarose gel at 110 volts for 45 minutes. 100-bp DNA ladders were used to identify the size of the PCR products, and the bands were visualized using Gel Doc (Vilber Lourmet). The reference gene used in the analysis was *GAPDH*, which served as a housekeeping gene. The relative expression of the

Table 1: Primers are utilized in PCR reaction.

	Primer name	Sequence	Product size
GAPDH gene	Forward primer	5-CTCCCAACGTGTCTGTTGTG-3	222bp
	Reverse primer	5-TGAGCTTGACAAAGTGGTCG-3	
MX1 gene	Forward primer	5-ACATGAAACGGAGTCCAAGG-3	449bp
	Reverse primer	5-TGCCAGGAAGGTCTATCAGG-3	
MX2 gene	Forward primer	5-AGGTCATGCAGAACCTCACC-3	654
	Reverse primer	5-TAATTTCCATGGCCTTCTGG-3	

MX1 and *MX2* genes was determined by comparing them to the expression of *GAPDH* gene.

Data obtained

Image J. software version 1.47 was used to analyze the Gel bands and obtain data. The data was analyzed through SPSS software. A one-way ANOVA and LSD test were applied to the data obtained. The relative expression of *MX1*, *MX2* and *GAPDH* was demonstrated.

Relative expression = gene data/*GAPDH*

Results

A total of 4 out of 6 ewe enrolled in this study became pregnant, and 1 remained non-pregnant as experimentally proved by *MX* genes expression, while 1 was kept as a control. The pregnant ewes were confirmed by ultrasound on day 30. *MX* genes were expressed from days 14 to 17. No expressions were recorded in non-pregnant or control ewe. **The housekeeping gene GAPDH, which has 222 bp, was used as a reference gene.**

MX1 and *MX2* gene Expression of Pregnant and Non-Pregnant ewe on specific days.

The gel bands show amplification results of **the MX1** and *MX2* genes and **GAPDH** on day 14 post-insemination (Figure 1(a-e)). In the given figure, C indicates control, N.P indicates non-pregnant, and P indicates pregnant ewes. The results showed the expression of the **MX1** and MX2 genes on day 14 only in pregnant ewes, as shown in lines 3, 4, 5, and 6 of the gel electrophoresis picture. The **GAPDH** gene was also successfully amplified in all samples (Figure 1b), which showed expression in all ewes on day 14. The bar graph shows the relative expression of the **MX1** and *MX2* genes on day 14 (Figure 1(d,e)). The samples were taken on day 16, and subsequent PCR amplification showed the expression of the **MX1** and *MX2* genes in all pregnant ewes, as shown in figure 1(f-j), but no expression was detected in the control and non-pregnant ewes on day 16. *GAPDH* gene were expressed in all samples (Figure 1g) and (Figure 1 (i,j)) represent the relative expression of *MX1* and *MX2* gene in day 16 Same as before. On day 17 (Figure 1(k-o) and 18 (Figure 1 (p-t)), the *MX1* and *MX2* genes expression was detected in pregnant ewes, but in non-pregnant ewes, the expression was not detected. **The GAPDH** gene showed full expression in all experimental ewe (Figure 1 (i,q)).

MX1 Expression on successive days

The bar graph shows the relative expression pattern of the **MX1** gene on days 14, 16, 17, and 18 post-inseminations (Figure 2). The expression was only detected in pregnant ewes, which was confirmed by ultrasonic examination of all ewes on day 30 post-insemination. But in the non-pregnant and control, no expression was observed. On day 17, the expression was detected higher as compared to other expression days. The Y-axis shows the relative expression of the **MX1/GAPDH** gene, while the x-axis shows the days of expression.

MX2 Expression on successive days

The graph shows the expression pattern of the **MX2** gene on days 14, 16, 17, and 18. Expression was detected only in pregnant ewes, but in non-pregnant and control ewes, no expression was observed (Figure 3). Like **MX1,** on day 17, the expression of the **MX2** gene was detected higher as compared to other expression days, but **MX2** gene expression on successive days was detected higher as compared to the **MX1** gene on successive days.

Comparison of relative expression of *MX1* and *MX2* between pregnant and non-pregnant ewes on different days

The relative expression pattern of the **MX1** and **MX2** genes on days 14, 16, 17, and 18, as I mentioned before, the expression was detected only in pregnant ewes, but in non-pregnant and control animals, no expression was observed. As shown in figure 4, on day 14, the expression of *MX1* is low as compared **to MX2.** Similarly, on the other days, **MX2** gene expression was relatively higher as compared to **MX1 gene expression.** Both genes showed significantly higher expression on day 17 as compared to other days. The expression pattern of both the genes was almost similar, starting from day 14 and then gradually increasing, reaching its peak on day 17 and then decreasing on day 18. The highest expression was detected by the *MX2* gene on day 17 (Figure 4).

Discussion

The present research is intended to investigate the expression pattern of *MX1* and *MX2* genes during the early pregnancy in ewes. The Kari breed (a local breed) of ewes was used for the study. The selection of the Kari breed was due to its short gestation period and the unavailability of early studies on this breed [30].
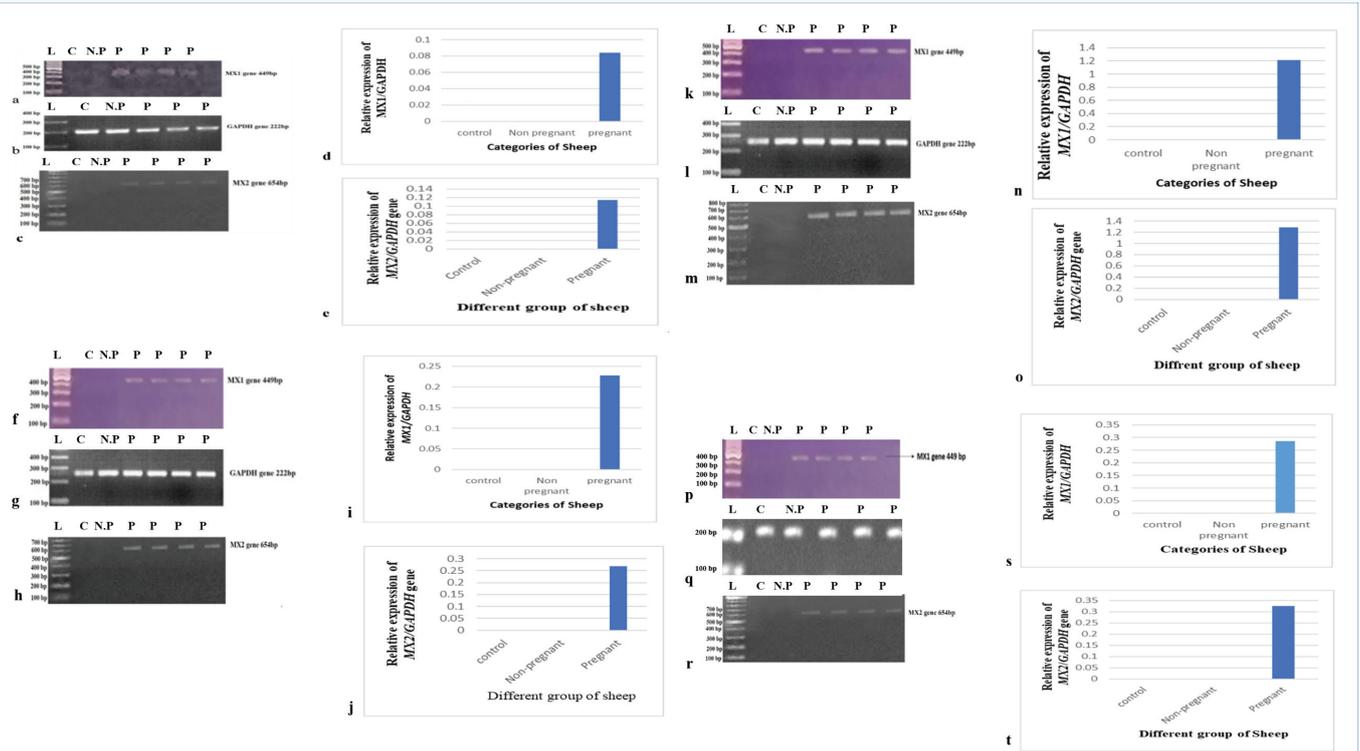
Figure 1 Represents the relative expression of *MX1/GAPDH* and *MX2/GAPDH* in pregnant and non-pregnant ewe. Also show the differences in expression pattern between the Pregnant and Non-pregnant ewe for each gene and each day post-natural insemination.
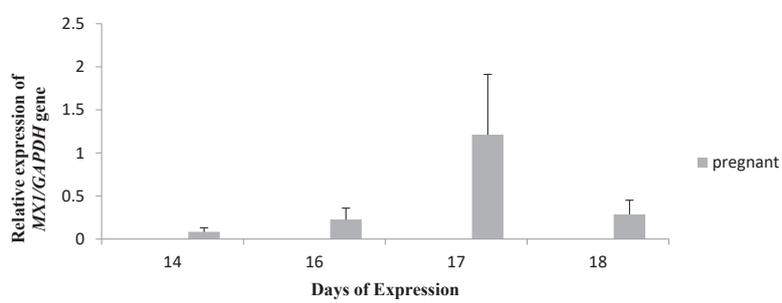

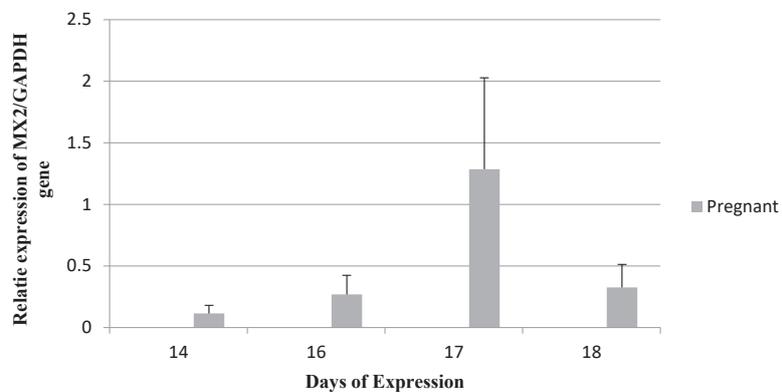**Figure 2** Expression of *MX1/GAPDH* gene on successive days.

Figure 3 The relative expression of *MX2/GAPDH* gene on successive days.

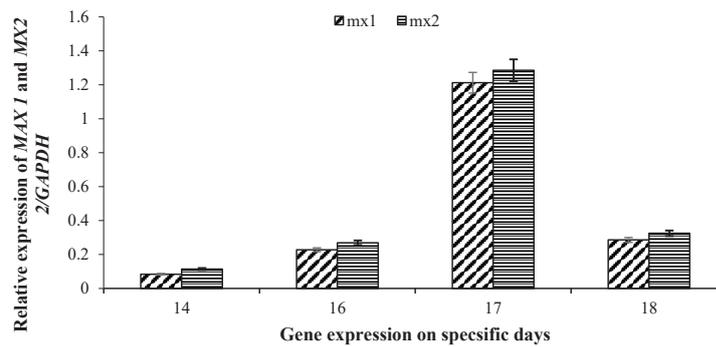Subject Area(s): GENETICS | MOLECULAR BIOMARKERS

Figure 4 Relative expression of *MX1* and *MX2* genes on successive days.

A total of six sheep were selected for the experiment, including one sheep kept as a control. Due to the institutional ethical committee guidelines, which accentuate on limiting the number of experimental animals, we only use one ewe as control. Blood was obtained from both experimental and control ewes. However, expression of *MX* genes was only found in pregnant ewes at days 14, 16, 17, and 18. While no expression was observed in non-pregnant ewes. The reason for the expression of the selected genes in pregnant ewes may be due to the fact that *MX* genes are interferon-stimulating genes, and interferon tau is released from trophoblast cells of the embryo only in pregnant ewes and stimulates the expression of many endometrial genes responsible for it. Maternal acknowledgment of pregnancy and progression is required. The expression of *MX-1* is linked to the progression of pregnancy. [31]. In similarity to our results [32], we found that the expression of *MX* genes is upregulated in pregnant ewes. Illustrated that the expression of *MX2* and *OAS1* was upregulated in pregnant cows compared to cycling cows [31]. Our results showed that expression of the *MX1* and *MX2* genes started on the 14th day of mating (Figure 1). Similarly, [33] found that expression of interferon-stimulating genes in ewes begins as early as day 12 of mating. Similarly, [34] reviewed *MX1* RNA levels in LE stroma and myometrium. The maximum RNA level was found on day 15, and RNA was detected as early as day 11. However, no expression of *MX1* mRNA was found in non-pregnant ewes. Another study by [35] examining the relative expression of *MX* RNA found that the expression of *MX* genes starts at day 14 of mating and peaks at day 17 after mating. Some of these studies reported differences in the day of first expression of *MX* genes from what we have reported. A possible explanation for this is that the breed of ewe

we used in our study (Kari ewe) is a non-seasonal breed. It is known that Kari ewe have a relatively shorter gestation period compared to other ewe breeds [30]. Therefore, non-seasonality and fluctuations in their gestation periods can be a possible reason for the different expression on certain days. Regarding the expression pattern of the *MX* genes, our results showed a change in the expression pattern in pregnant and non-pregnant ewes. Expression of the *MX1* and *MX2* genes in pregnant ewes begins on day 14 and peaks on day 17, decreasing slightly from day 18 until completely disappearing on day 20. On the other hand, expression was not observed on any of the test days in non-pregnant ewes. In the study [21]], synthetic interferon-tau was administered intramuscularly to examine its effect on the gene expression of *MX2* and *ISG15* in bovine peripheral blood mononuclear cells. The results of the study showed that there is a correlation between the pattern of gene expression and the interferon administered. The study also linked embryonic deaths to the expression pattern of genes. The study concluded that INF-tau amounts can be estimated from *ISG15* and *MX* gene expression in PBMCs. The differences in the expression pattern of MX genes in these studies and in our studies may be due to the experimental ewe used in this study having shorter gestation periods. Since *MX* genes are interferon-stimulating genes and interferon tau is released by the dividing term, these differences in pattern may be due to the use of different breeds of ewe. In the case of non-pregnant ewes, since there was no release of interferon, *MX* genes were not expressed, and therefore their expression was not recorded in non-pregnant ewes. From this study, it is concluded that in pregnancy, *MX1* and *MX2* are expressed due to the release of interferon tau from the newborn. Because these genes play an antiviral role, they

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are actually targeted against negative–strand RNA viruses. Their expression is observed in those cells that have INT receptors and are normally prognostic for viral attacks. Once the ewes are conceived, these genes begin to be expressed by interferon tau. The study also suggests that the *MX* genes could be used indirectly for early detection of pregnancy in ewes.

Conclusion and Recommendation

The **MX1** and **MX2** genes were particularly expressed in pregnant ewes. No expression was observed in non–pregnant and control ewes. Expression of the **MX** genes was at its maximum on day 17. Expression of the *MX* genes started their expression from day 14, while expression down regulated after day 17. Moreover, *MX2* gene expression was recorded higher as compared to *MX1* gene. In addition, a larger research sample size is required to fully explore the role of the *MX* genes. A comparative expression analysis of the gene should be carried out in different ewe breeds. It is necessary to find other ways to detect early pregnancy in order to reduce the loss during the open period.

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Author's Contributions

TK conceptualized the study, designed the experiment, and performed manuscript writing. MS performed the statistical analyses and drew the graphs. IH and SH's overall guidance, read the study. GZ approved and submitted the final manuscript.

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Declarations

The research article submitting "Expression analysis of MXI and MX2 genes for maternal recognition of concept us in ewes" for publication in your journal of repute is a unique article, and nobody did it earlier.

Consent for Publication

Consent was obtained from all participants for this publication.

Competing Interests

The authors declare that they have no competing interests.

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