“H19 GENE EXPRESSION STUDIES IN PREGNANT AND NON PREGNANT GOATS”

THESIS
Submitted to the

Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya,

Jabalpur

In partial fulfilment of the requirements for

the Degree of

MASTER OF VETERINARY SCIENCE AND

ANIMAL HUSBANDRY

In

ANIMAL BIOTECHNOLOGY

By

SUNIL KUMAR

ANIMAL BIOTECHNOLOGY CENTER
Madhya Pradesh Pashu Chikitsa Vigyan Vishwavidyalaya,

Jabalpur (M.P.)

2012
1. INTRODUCTION

Goat is known as ‘Poor man’s cow’ in India and is a very important component of dry land farming system. For marginal or undulating lands unsuitable for other types of animals like cow or buffalo, goat is the best alternative. With very low investments goat rearing can be made into a profitable venture for small and marginal farmers.

Early detection of pregnancy aids in culling or rebreeding of barren does, and provides a valuable tool for controlled breeding programs. Inability to detect early pregnancy can result in economic losses in milk and kid production due to longer kidding intervals. In larger domestic animals such as the cow, buffalo and horse, palpation of the genital tract per rectum facilitates the early diagnosis of pregnancy which is not possible in the smaller farm animals.

Many methods have been described to diagnose pregnancy in goats. These include ultrasonography (A-scan Watt et al., 1984), Doppler ultrasonic (Trapp and Slyter, 1983), Real time B-scope ultrasonics (Davey, 1986; Haibel, 1990; Bretzlaff et al., 1993), Estrone sulfate (Tsang, 1978; Refstal et al., 1991), progestrone (Murray and Newstead, 1988), radiography (Barker and Cawley, 1967), rectal-abdominal palpation (Ott et al., 1981), vaginal biopsy (Richardson, 1972a), abdominal palpation and ballotment (Pratt and Hopkins, 1975), palpation of the uterus via laparotomy (Smith, 1980) and pregnancy specific antigen (Ruder et al., 1988).

The H19 gene is a parenterally imprinted maternally expressed gene which has a pivotal role in embryogenesis and fetal development. Ever since, H19 gene was first identified in the mouse as a foetal-specific mRNA expressed under the control of raf, a transacting locus gene that determines the adult basal level of α-fetoprotein (AFP) in the liver (Pachnis et al., 1988), interest to identify this gene in the embryonic tissues took a momentum. However, expression of H19 in the extra embryonic membranes was detected only after the ovine conceptus began attachment to the endometrium and the
embryo itself had undergone early organogenesis (Lee et al., 2002) that may be regarded as the first step in implantation.

Ayesh et al (2002) reported the association of H19 gene with the regulation of cellular invasion, migration and angiogenesis. Further, the author also reported that dynamic changes in H19 imprinting may be related to formation of placenta and maintenance of normal pregnancy. It was further demonstrated that angiogenesis factors and TNF-α and NFκB are regulated by H19 gene.

Ever since, H19 gene was first identified in the mouse as a foetal-specific mRNA expressed under the control of raf, a transacting locus gene that determines the adult basal level of α-fetoprotein (AFP) in the liver (Pachnis et al., 1988), interest to identify this gene in the embryonic tissues took a momentum. However, expression of H19 in the extra embryonic membranes was detected only after the ovine conceptus began attachment to the endometrium and the embryo itself had undergone early organogenesis (Lee et al., 2002) that may be regarded as the first step in implantation.

Implantation is a critical step in the progress of pregnancy, during which the conceptus acquires a fixed position within the uterine lumen, and leads to the establishment of the placental structures. In ruminants, the implantation process is characterized by a long pre-attachment period lasting 2-3 weeks during which the conceptus elongates considerably, an apposition stage when cellular contacts are established between the trophoblast and the uterine epithelium, and an adhesion stage which ends the process and gives rise to the cellular structure of an epithelio-chorial placenta. Trophoblast apposition begins in the vicinity of the embryo by day 15, 18 and 19 in sheep, goats and cows, respectively. Apposition is completed by a close adhesion between the interdigitating uterine microvilli and the trophoblastic plasma membrane. By this stage, trophoblastic binucleate cells migrate through the trophoblast monolayer and fuse with individual uterine cells to form a syncytial tissue. During this process placental lactogen hormones and pregnancy serum proteins (PSP) produced by the binucleate cells are transported to the
endometrial tissues and then to the maternal blood circulation (Guillomot, 1995).

By the time adhesion between the uterine microvilli and foetal trophoblastic plasmamembrane is established the process of angiogenesis has already started allowing the exchange of micro and macro molecule between fetal and maternal blood. The H19 gene expression due to its role in that regulation an untranslated RNA can be detected in maternal blood, Immediatley after early embryogenesis around 15-17 days hence, the expression H19 gene in maternal blood can be taken as index for early pregnancy diagnosis.

In a preliminary study in the present laboratory using maternal blood it was observed that H19 gene is expressed in maternal blood circulation in pregnant goats but not in the non pregnant goats (Annonymous., 2011). However, there is no published report of pregnancy diagnosis in any mammalian species using H19 gene expression. this prompted us to undertake a planned research to correlate H19 gene expression an index to diagnose the pregnancy in goats. Therefore, the present investigation has been designed with following objectives.

1. Amplification of H19 gene in pregnant goat.
2. Attempt to diagnose early pregnancy with the help of H19 gene expression.
2. REVIEW OF LITERATURE

Establishment of pregnancy involves maternal recognition of pregnancy and implantation. It can be defined as the physiological process whereby the conceptus signals its presence to the maternal system and prolongs lifespan of the corpus luteum (CL). Progesterone production by the CL is required for successful pregnancy. Progesterone acts on the uterus to stimulate and maintain uterine functions that are permissive to early embryonic development, implantation, placentation and successful fetal and placental development to term. Prolonged lifespan of the CL is a characteristic feature of mammalian pregnancy in species with a gestation period that exceeds the length of a normal estrous cycle. Maintenance of pregnancy requires reciprocal interactions between the conceptus and endometrium. Available evidence supports the idea that hormones from the placenta act directly on the uterine endometrium to regulate cell differentiation and function. In domestic animals, the endometrial glands undergo a program of hyperplasia followed by hypertrophy that appears to be dependent on temporal and spatial actions of hormones from the placenta. Endometrial gland morphogenesis during pregnancy allows for the endometrium to increase output of secretory proteins that are transported to the fetus by specialized areas of the placenta termed areolae. Histotrophic nutrition from the endometrium is the first available nutrition for the developing conceptus and appears to be essential for conceptus survival and growth throughout pregnancy in domestic animals (Spencer and Bazer, 2004).

Implantation is a critical step in the progress of pregnancy, during which the conceptus acquires a fixed position within the uterine lumen, and leads to the establishment of the placental structures. This process implies some cellular modifications of both the uterine epithelium and the trophoblast to ensure cell adhesion between the two tissues. In ruminants, the implantation process is characterized by three main steps: a long pre-attachment period lasting 2-3 weeks during which the conceptus elongates considerably, an apposition stage when cellular contacts are established between the trophoblast and the uterine epithelium, and an adhesion stage.
which ends the process and gives rise to the cellular structure of an epithelio-chorial placenta. Trophoblast apposition begins in the vicinity of the embryo by day 15, 18 and 19 in sheep, goats and cows, respectively. The trophoblast cells surrounding the embryo show morphological and functional changes. These modifications are local within the conceptus since non-implanted areas of trophoblast still display the morphological and functional features that characterized this tissue during the pre-attachment period. As the implantation process spreads towards the extremity of the elongated conceptus, these cellular transformations progressively affect the whole trophoblast. Apposition is completed by a close adhesion between the interdigitating uterine microvilli and the trophoblastic plasma membrane. By this stage, trophoblastic binucleate cells migrate through the trophoblast monolayer and fuse with individual uterine cells to form a syncytial tissue. During this process placental lactogen hormones and pregnancy serum proteins (PSP) produced by the binucleate cells are transported to the endometrial tissues and then to the maternal blood circulation (Guillomot, 1995).

2. **Pregnancy diagnosis in sheep and goats**

Reliable techniques for early detection of pregnancy aids in culling or rebreeding of barren ewes and does, and provides a valuable tool for controlled breeding programs. Inability to detect early pregnancy can result in economic losses in milk and kid production due to longer kidding intervals. In larger domestic animals such as the cow and horse, palpation of the genital tract per rectum facilitates the early diagnosis of pregnancy which is not possible in the smaller farm animals.

Traditional methods of visual observation, abdominal palpation, service records and non-return to estrus are not reliable means of diagnosing early pregnancy. Non-return to estrus following breeding may be suggestive of pregnancy, but pathological conditions of the uterus or ovaries, physiological anestrus late in the breeding season and out of breeding season may cause an estrus in nonpregnant ewes and does.

Non-return to estrus is an unreliable method when ewes or does are synchronized and bred during the non-breeding season. Many
methods have been described to diagnose pregnancy in goats, however, most techniques are not adaptable to field conditions. These include ultrasonography (A-scan Watt et al., 1984); Doppler ultrasonic (Trapp and Slyter, 1983); Realtime, B-scope ultrasonics (Davey, 1986; Haibel, 1990; Bretzlaff et al., 1993); Estrone sulfate (Tsang, 1978; Refstal et al., 1991); progestrone (Murray and Newstead, 1988); radiography (Barker and Cawley, 1967); rectal abdominal palpation (Ott et al., 1981) vaginal biopsy (Richardson, 1972a); abdominal palpation and ballotment (Pratt and Hopkins, 1975); palpation of the uterus via laparotomy (Smith, 1980); pregnancy specific antigen (Ruder et al., 1988).

2.1 Ultrasonic techniques

Ultrasonic techniques can be used to examine subsurface structures in living tissues by use of A-scan or Doppler techniques. Ultrasound is reflected from moving tissues, such as blood at a slightly changed frequency. One of the most important features of ultrasound, when used for tissue examination, is its safety to the operator and patient (Bishop, 1966). Pregnancy may be detected in does with all three types of ultrasonographicals available, amplitude depth (A-scan), Doppler and Real time B-scan ultrasonics. Each can be used under field conditions. The accuracy of diagnosis, timing of examination, fetal numbers and age and fetal viability vary considerably among these techniques.

2.1.2. A-Scan ultrasonic techniques

A light or audible signal is emitted by the unit or characteristic blip patterns displayed on an oscilloscope when a fluid filled structure is detected. A-scan ultrasound applied to the flank region has proven to be reasonably reliable from 50 to 120 days of gestation in sheep and goats (Wani, 1981; Watt et al., 1984). An extended urinary bladder, hydrometra or pyometra may give false positives. False negatives may occur in early gestation or in late gestation because of decrease in the ratio of uterine fluid to fetal tissue. Neither fetal viability nor fetal numbers are detected by this method. Early work with externally applied A-scan indicated that an accuracy of at least 95% is possible between 60 and 80 days of gestation in ewes.
(Haibel, 1990). However, Meredith and Madani (1980) reported that a positive diagnosis of pregnancy in ewes can be made on the evidence of ultrasound reflections with an accuracy of 83% 61 to 151 days after mating. Lindahl (1969a,b) reported that the earliest time at which pregnancy could be detected by using A-scan is between 40 and 50 days after mating.

2.1.3. Doppler ultrasonics

The principles involved in Doppler ultrasonics for diagnosis of pregnancy is the detection of movements as an indication of pregnancy such as fetal heart beat, fetal circulation and fetal movement It detects maternal fetal tissue interfaces (Wani, 1981; Trapp and Slyter, 1983; Watt et al., 1984). ultrasonic Doppler has been used for detection of pregnancy in ewes and does) and approaches an accuracy of 100% during the last half of gestation (Fraser and Robertson, 1968; Lindahl, 1968; Keane, 1969; Shone and Fricker, 1969) but is not effective 50 days or earlier (Lindahl, 1969a,b, 1971).

2.1.4. Doppler ultrasonics - rectal

Compared to A-scan technique, the Doppler technique resulted in greater accuracy in ewes, which were at least 65 days pregnant (Lindahl, 1969). The intra-rectal Doppler technique also allows detection of pregnancy earlier in gestation than the A-scan technique (Lindahl, 1971).

2.1.5. B-Scan ultrasonic techniques

Ultrasonic techniques based on the Doppler principle (Lindahl, 1971) can diagnose multiple pregnancies with an acceptable accuracy Transabdominal scanning is similar in ewe or doe. The ideal time for transabdominal scanning is between 40 to 75 days of gestation.

2.2. Hormone assay

Measurement of concentrations of steroid hormones such as Estrone sulfate and progesterone at specific times post-breeding provide another method of pregnancy diagnosis in small ruminants (Tsang, 1978; Tamanini et al., 1986; Worsfold et al., 1986; (Bretzlaff et al., 1989). Plasma
concentrations of progesterone tend to be more accurate than milk (Bretzlaff et al., 1989). Multiple versus single birth predictions were only 67.4% accurate owing to a wide variation in the concentration of progesterone (Weigh et al., 1975).

2.3 Rectal abdominal palpation

Rectal abdominal palpation technique for diagnosing pregnancy in the ewe was first described by Hulet (1972). This technique has also been used in the doe (Ott et al., 1981). Rectal abdominal palpation is simple, quick, accurate and inexpensive.

2.4 Radiography

The technique of radiography can be used to detect pregnancy and multiple birth with an accuracy of 90% or more.

2.5 Vaginal biopsy

Histological evaluation of vaginal biopsies have an accuracy of 97% for diagnosing pregnancy in ewes pregnant for more than 40 days (Richardson, 1972a).

2.6 Palpation of uterus via laparotomy

The gravid uterus can be palpated directly through a small incision in the abdominal wall. Direct palpation of the uterus gave more than 92% accuracy in diagnosing pregnancy in ewes 4-5 weeks pregnant (Hulet and Foote, 1968) whereas in does it approaches 100% after 42 days of gestation (Smith, 1980).

2.7 Abdominal palpation and ballotment

Ewes and does in the late stages of pregnancy can be examined by these techniques. These become easier and more reliable as pregnancy advances. It is easier in thin ewes and does than in fat animals. Pratt and Hopkins (1975) recorded accuracies of 80 to 90% in ewes 90-130 days of pregnancy.
2.8. Detection of pregnancy-specific antigen

Detection of the pregnancy-specific antigen, chorionic somatotrophin in serum was utilized for pregnancy diagnosis in a commercial flock of 286 sheep 70 days after joining with rams (Robertson et al., 1980). Ewes were pregnant between Days 47 and 70. Based upon lambing results the positive diagnosis was 97% correct. This test can be successfully used after Day 55 of pregnancy. Concentration of ovine chorionic somatotrophin is measured with radioimmunoassay.

2.9 Palpation of the cervix

This technique involves digital palpation of the external cervical os per vaginum at 50 days or more post-breeding. A very soft, blunted cervix or inability to reach the cervix is suggestive of pregnancy while a firm conical-shaped cervix projecting into the vagina is suggestive of nonpregnancy (Richardson, 1972b).

2.10. Mammary secretion

Webb (1942) found that ewes carrying their first lambs produced a sticky honey-like mammary secretion after the third month of gestation, and multiparous ewes produced a more watery secretion. Richardson (1972a) tested 11 ewes at 30-80 days of gestation and the stickiness was not observed.

2.11 Pregnancy associated glycoprotein (PAG)

The PAG milk-test provides an accurate pregnancy diagnosis from Day 32 after breeding and, in combination with good management practices, this test would be suitable under farm conditions to confirm pregnancies tentatively identified by nonreturn to estrus on Day 21 after breeding. Likewise, carrying out this test on Day 35 of pregnancy or later could provide a predictor for ongoing pregnancy or pregnancy failure (Gonzalez et al., 2000).
2.12 Description of H19 gene

H19 is gene for a noncoding RNA, found in humans and other species. This gene has role in some forms of cancer (Ariel et al., 2000). The H19 gene is also known as Adult skeletal muscle (ASM). H19 was first named ASM because of its expression in adult skeletal muscle in the rat (Leibovitch et al., 1991). This gene encodes a sequence that is not translated and expressed on one parental allele in a phenomenon known as imprinting (Zhang et al., 1992). H19 is transcribed exclusively from the maternally inherited allele; the paternal H19 allele is not expressed (Bartholdi et al., 2009).

2.13 Structure and mapping of H19 gene

The human H19 gene is 2.7 kb long and includes 5 exons and 4 small introns (Brannan et al., 1990). Leibovitch et al. (1991) mapped the human gene to 11p15. In the mouse, the H19 gene is located on chromosome 7 in a region of conservation of synteny with human 11p (Jones et al., 1992). In cattle and sheep H19 gene is located on chromosome 29 and 21, respectively. Like the H19 gene, the IGF-2 gene is imprinted in the mouse, although in the opposite parents, one paternally imprinted, the other maternally. IGF-2 gene lies about 90 kb 5′-prime to H19. Both genes hybridized to a fragment of about 200 kb (Zemel et al., 1992). H19 gene codes for a capped, spliced and polyadenylated RNA. Particularity of this product is its apparent inability to be translated when the UTR 5′ is not experimentally altered (Brannan et al., 1990; Joubel et al., 1996). As a consequence, it has been proposed that this gene could act as a `riboregulator' (Brannan et al., 1990). Recently, Li et al. (1998) proposed that H19 RNA is associated with polysomes and modulates the cytoplasmic level of IGF-2 mRNAs.

2.14 Importance of H19 gene

Mutter et al. (1993) found that normal gestations express H19 only from the maternal allele. The H19 gene was first identified in the mouse as a foetal-specific mRNA expressed under the control of raf, a transacting locus gene that determines the adult basal level of α-fetoprotein (AFP) in the
liver (Pachnis et al., 1988). Subsequently, it was found to be highly expressed during mouse foetal development in non-hepatic tissues that normally do not express AFP, such as skeletal and cardiac muscle. The mouse cDNA was also isolated independently (Davis et al., 1987) as a gene called myo H that was highly expressed during the differentiation of myogenic precursors, to myoblasts and later, as a gene activated during embryonic stem cell and embryonal carcinoma cell differentiation (Poirier et al., 1991). The extraordinary high levels of mRNA found in some tissues only during foetal development and the inability to detect a protein product for the gene prompted the cloning of the human homologue (Brannan et al., 1990).

H19 genomic imprinting is surmised to have arisen due to the conflicting interests of maternal and paternal genes within a pregnancy (Moore et al., 1991). H19 is highly expressed prenatally and downregulated postnatally (Banet et al., 2000). Postnatally, H19 is expressed at high levels in cancer cells (Brunkow and Tighman, 1991). In the early placentae (6–8 weeks gestation), both parental H19 alleles (maternal and paternal) are expressed. After 10 weeks gestation and in full term placentae, there is exclusive expression of H19 from the maternal chromosome (Arima et al., 1997).

The H19 gene in mice is maternally imprinted and its ectopic expression causes prenatal lethality. H19 transcript in differentiating human placental cells and showed that its expression increases concomitantly with differentiation of cytotrophoblasts in vitro. Placental and embryonal specimens were collected from conception products derived from normal first and second trimester pregnancy terminations. The abundance of H19 mRNA throughout placental development in vivo and compared it to the expression of other genes linked to placental differentiation. The expression of H19 transcript in different organs of human fetuses, aborted during the second trimester, was examined by RNA isolation from separated fetal organs (Goshen et al., 1993).

2.14.1 H19 gene in relation to pregnancy

The H19 gene is a parenterally imprinted maternally expressed gene which has a pivotal role in embryogenesis and fetal development. Ayesh et al (2002) reported the association of H19 gene with the regulation of
cellular invasion, migration and angiogenesis. Further the author also reported that dynamic changes in H19 imprinting may be related to formation of placenta and maintenance of normal pregnancy. It was further demonstrated that angiogenesis factors and TNF-α and NFκB are regulated by H19 gene (Yu et al., 2009).

The expression of H19 in the extra embryonic membranes was detected only after the ovine conceptus began attachment to the endometrium and the embryo itself had undergone early organogenesis (Lee et al., 2002). This may be regarded as the first step in implantation; thus, in comparison with the mouse, the initiation of H19 expression appears to be determined by the timing of implantation rather than by the stage of development of the embryo itself. H19 is one of the most important genes that plays a crucial role in development, differentiation and regulation of cell multiplication. It is a paternally imprinted gene that encodes an untranslated RNA. It is abundantly expressed in early stages of embryogenesis and fetal growth.

H19 is expressed in most fetal tissues, except in the thymus, and is repressed in almost all adult organs except the cardiac and skeletal muscles (Pachnis et al., 1988; Leibovitch et al., 1991), the uterus (Ariel et al., 1997b), to a lesser extent the kidney, the adrenal glands, the lung (Zhang and Tycko, 1992) and the mammary gland. This pattern of expression suggests that H19 could be implicated in fetal development. H19 could also intervene in postnatal development when the concerned organ continually undergoes repair, as for instance is the case with the mammary gland or the uterus.

The expression of the H19 gene has been well studied in fetal human and mouse tissues. It is generally believed that H19 is abundantly expressed in the early stages of embryogenesis and is repressed postnatally (Khatib and Schutzkus, 2006).

H19 imprinted gene might affect implantation and that their timely and appropriate activation is important for proper functioning. In human being the biallelic expression of the H19 gene exists in some cases at the early stage of normal pregnancy and changes into monoallelic expression
near 10 weeks of gestation. The dynamic alternations in the patterns of the H19 gene imprinting may regulate the maintenance of normal pregnancy (Yu et al., 2009).

2.14.2 Association of H19 and IGF-2 gene during development

The H19 gene is a parenterally imprinted maternally expressed gene which has a pivotal role in embryogenesis and fetal development. It is tightly linked to the IGF-2 gene on chromosome 11p 15.5 which is reciprocally imprinted (Lusting et al., 2005). The mouse H19 gene is located in the most 30 position of a cluster of genes whose expression is determined by the parental inheritance of the allele parental or genomic imprinting. Located between 90 and 100 kb 50 of the H19, gene in this cluster is the insulin-like growth factor II (IGF-2) gene, whose expression during embryonic and foetal development is tightly linked to that of H19. However, H19 is expressed from the maternal allele whereas IGF-2 is expressed from the paternal allele in most foetal tissues. The chromosomal arrangement and location of at least three imprinted genes, INS-2, IGF-2 and H19 in this cluster, is conserved in both the mouse and human.

H19 is more likely to be a marker for embryonic or foetal differentiation rather than a tumour suppressor gene. Recently it was proposed that H19 RNA may also act in trans to regulate IGF-2 expression by modulating the translatability of the IGF-2 mRNA, thus regulating the levels of IGF-2 produced by the cell (Li et al., 1998).

H19, IGF-2 and its receptor are three frequently studied imprinted genes involved in regulating early fetal growth and are essential for normal development (Basong et al., 2007). H19 is also expressed in several tumour types arising from tissues that normally express the gene during foetal development and in the regenerating liver (Pachnis et al., 1984).
3. MATERIAL AND METHODS

The study was conducted in two phases:

1. Expression of H19 gene in known pregnant and non-pregnant goats.
2. Expression of H19 gene as an index for early diagnosis of pregnancy in goats.

Phase 1

Studies on expression of H19 gene in known pregnant and non pregnant goats

Blood samples from twelve goats (6 non-pregnant and 6 pregnant) were collected at random and studied for expression of H19 gene. Pregnant animal were selected on the basis of abdominal palpation and further confirmed by ultrasonography to assess the approximate duration of pregnancy. Non pregnant animal were selected on the basis of cyclicity and judged by overt symptoms of oestrus.

Phase 2

Studies on expression of H19 gene as an index for early diagnosis of pregnancy in goats

Goats expressing symptoms of oestrus in a flock were isolated and mated with a buck of known fertility. Sequential blood samples were obtained from all the mated goats up to day 30 starting from day 15 following natural service at three days interval. All blood samples were analysed for expression of H19 gene. Alternatively, some samples of goats mated following induced oestrus were also obtained and studied. The animals following mating were observed for return to oestrus. All such animals were apparently classified as non-pregnant although the samples were continued to be collected up to day 30. Pregnancy was confirmed by day 60 following mating by abdominal palpation and/or ultrasonography. On classification of goats in pregnant and non-pregnant, their respective H19 gene expression results were tallied for an early diagnosis of pregnancy based on H19 gene expression.
3.1 Blood sampling for H19 gene expression studies

5 ml blood was collected from pregnant and non pregnant goat in EDTA coated vacutainer tubes.

3.2 Isolation of Total RNA from blood

Glasswares, plasticwares and water used for RNA isolation were first treated with 0.01% DEPC for an overnight duration to make them nuclease free. Glasswares and plastic wares were then taken out from DEPC treated water wearing hand gloves and dried at 60ºC in hot air oven. DEPC treated water, glasswares and plasticwares were then autoclaved at 15 lbs pressure for 20 min for inactivation of DEPC.

RNA isolation was done by using TRIZOL (Sigma, U.S.A) reagent as per manufacturer’s instructions with some modifications.

Steps-

1. The contents of tube were transferred into a 50 ml polypropylene conical centrifuge tube.

2. Volume was brought to 50 ml with RBC lysis buffer.

3. The contents were kept at room temperature for 10 to 15 minutes or for 20 to 30 minutes at 4°C.

4. The cells were pelleted at 600 x g (approx 1,400 rpm) for 10 minutes in a 4°C centrifuge.

5. The supernatant was carefully decanted.

6. The pellet was gently resuspended in 1 ml of RBC lysis buffer and transferred to a 1.5 ml microcentrifuge tube. Kept for 2 to 5 minutes.

7. The cells were pelleted for 2 minutes by centrifuging in a microfuge at 3000 rpm at room temperature.

8. The supernatant was carefully aspirated.

9. If the resulting pellet was still red, the RBC lysis steps were repeated as needed.

10. The pellet was resuspended in 1 ml of sterile PBS.
11. The cells were pelleted as in step 10.
12. The supernatant was carefully aspirated.
13. The cells were processed for RNA if desired (or stored cells at -80°C until needed). The cells were chosen for the pellet was not thawed prior to RNA isolation.
14. 800 µl of Trizol/RNA stat 60 solution was added to each tube and the cells were resuspended.
15. 0.2 ml of Chloroform (CHCl₃) was added and each tube was vortexed for 15 seconds, one at a time.
16. The samples were kept on ice for 5 to 10 minutes.
17. The samples were centrifuged at 14,000 rpm for 15 minutes at 4°C.
18. The upper phase was removed and transferred to a clean micro centrifuge tube to which an equivalent volume of ice-cold isopropanol was added. Care was taken not to remove any of the white interface while collecting the upper phase of the extraction. The content were immediately vortexed and placed on ice for 15 minutes. The samples were stored at -20°C for further processing.
19. The samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. A small white pellet of RNA was seen at the bottom of the tube after this step.
20. The supernatant was carefully decanted and the pellet was twice rinsed with 0.5 ml of ice-cold 75% ethanol to remove salt.
21. The samples were centrifuged 7500 rpm for 8 minutes at 4°C. The supernatant was decanted.
22. Quick spinning of the sample was done at 14000 rpm for 30 seconds at 4°C to drive the remaining fluid of the bottom of the tube.
23. All of the remaining liquid at the bottom of the tube was carefully removed with help of p10 pipettor.
24. The pellet was allowed to dry for 5 to 10 minutes to remove any remaining ethanol.
25. RNA pellet was air dried and then dissolved in 50 µl DEPC treated water by incubating in water bath at 65°C for 10 min. Concentration of the total RNA was determined by spectrophotometer (OD at 260). The extracted RNA was further used for synthesis of cDNA or stored at -20°C until further use. 1200 ng RNA was used for cDNA synthesis for each sample of pregnant and non pregnant and early pregnancy goats.

26. Yes DNAase treatment done for removing DNA contamination.

3.2.1 cDNA preparation and quantification

cDNA was prepared from 12 µl (6 µl× 2 reactions for each sample) of RNA using Revert Aid™ first strand cDNA Synthesis kit (Fermentas) as per manufacturer’s instructions. All the glasswares and plasticwares used for cDNA preparation were made nuclease free before use. In a total 20 µl of reaction mixture, the components were added in the following order:

<table>
<thead>
<tr>
<th>DEPC treated water</th>
<th>5µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random hexamer primer</td>
<td>1 µl</td>
</tr>
<tr>
<td>RNA template</td>
<td>6 µl</td>
</tr>
<tr>
<td>5X reaction buffer</td>
<td>4 µl</td>
</tr>
<tr>
<td>RiboLock™ RNase inhibitor</td>
<td>1 µl</td>
</tr>
<tr>
<td>10mM dNTP mixture</td>
<td>2 µl</td>
</tr>
<tr>
<td>RevertAid™ M-MuLV reverse transcriptase (200 unit/ µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

An RT-PCR reaction containing all components except reverse transcriptase was also prepared as a negative control.

3.2.2 Reaction protocol for cDNA synthesis

<table>
<thead>
<tr>
<th></th>
<th>Step-I</th>
<th>Step-II</th>
<th>Step-III</th>
<th>Step-IV</th>
<th>Step-V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>65°C</td>
<td>25°C</td>
<td>42°C</td>
<td>70°C</td>
<td>4°C</td>
</tr>
<tr>
<td>Time</td>
<td>5 min.</td>
<td>5 min.</td>
<td>60 min.</td>
<td>5 min</td>
<td>∞</td>
</tr>
</tbody>
</table>
3.2.3 GAPDH (housekeeping gene) RT-PCR for checking cDNA integrity

1. The cDNA was generated with the control first strand synthesis.
2. The content were gently vortexed and all PCR reagents after thawing were briefly centifuged.
3. A thin-walled PCR tube was placed on ice and the following reagents were added.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>2 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X PCR master mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Water, Nuclease-free</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Forward GAPDH Primer</td>
<td>1 µl (10 picomole/µl)</td>
</tr>
<tr>
<td>Reverse GAPDH Primer</td>
<td>1 µl (10 picomole/µl)</td>
</tr>
<tr>
<td>Total Volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

4. RT-PCR was performed in Thermal cycler with a heated lid or overlaid with 25 µl of mineral oil.

<table>
<thead>
<tr>
<th>Step</th>
<th>temperature °C</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58</td>
<td>30 s</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>45 s</td>
<td></td>
</tr>
</tbody>
</table>

5µl of the RT-PCR product and 1µl 6X loading dye mix of each sample was loaded on 2% agarose gel. Visibility of a distinct 496 bp PCR product was ensured after Ethidium bromide staining. Constant voltage of 5-8 V/cm. for (approx.) 50-60 min at 37°C in 0.5 X TBE buffer was used. The suitable 100 bp DNA ladder was used as marker. The gels were photographed using an ultraviolet transilluminator (Gel Doc system). After checking integrity of cDNA of pregnant, non pregnant, and early pregnancy goats. These cDNA were used for H19 gene amplification. PCR amplification in pregnant, non pregnant and early pregnancy goats cDNA were done by using specific published primer. Annealing temperature was 52°C and working primer concentration was 10 picomole/µl (stock solution concentration 500 picomole/µl). Amplification product obtained was of 133 bp from cDNA.
3.2.4 Primer

H19 published Primer was used in this study (Basong et al., 2007).

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequences of primer 5’-3’</th>
<th>Size of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19 Forward</td>
<td>GGACTGGAACCTGGACTTCTTCA</td>
<td>23 Mer</td>
</tr>
<tr>
<td>H19 Reverse</td>
<td>TGGGTGTGGGTCTTCCGTTC</td>
<td>19 Mer</td>
</tr>
</tbody>
</table>

3.2.5 RT-PCR with H19 Primer for amplification of H19 gene

1. The cDNA was generated with the control first strand synthesis.
2. The cDNA was gently vortexed and all PCR reagents were briefly centrifuged after thawing.
3. A thin-walled PCR tube was placed on ice and the following reagents were added.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>2 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X PCR master mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Water, Nuclease-free</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Forward H19 Primer</td>
<td>1 µl   (10 picomole/µl)</td>
</tr>
<tr>
<td>Reverse H19 Primer</td>
<td>1 µl   (10 picomole/µl)</td>
</tr>
</tbody>
</table>

4. RT-PCR was performed in thermal cycler with a heated lid or overlaid with 25 µl of mineral oil.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>52</td>
<td>1 min</td>
<td>31</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>
3.2.6. Agarose gel Electrophoresis

5µl of the RT-PCR product and 1µl 6X loading dye mix of each sample was loaded on 2% agarose gel. Visibility of a distinct 133 bp (Amplicon size) PCR product was ensured after Ethidium bromide staining in pregnant non pregnant goats and early pregnancy goats samples. Constant voltage of 5-8 V/cm. for (approx.) 50-60 min at 37°C in 0.5 X TBE buffer. The suitable 100 bp DNA ladder was used as marker. Gels were photographed using an ultraviolet transilluminator (Gel Doc system).
4. RESULTS

The study was conducted in two phases:

1. Expression of H19 gene in known pregnant and non-pregnant goats.

2. Expression of H19 gene as an index for early diagnosis of pregnancy in goats.

H19 gene expression was studied by amplification in PCR on 2% agarose gel run. In pregnant animals, amplification of H19 gene by PCR was of 133 bp in size. However, in non-pregnant animals, the H19 gene was not amplified by PCR on gel documentation system.

**Phase 1**

**Studies on expression of H19 gene in known pregnant and non pregnant goats**

Twelve goats (6 non-pregnant and 6 pregnant) were studied for expression of H19 gene. The results are presented in table 1.

It is evident from the table that 6 goats viz. No. 10, 14, 16, 65, 413 and 52 that were in the pregnancy range from 2½ to 3½ months as evidenced by abdominal palpation and/or ultrasonography. Blood samples of all these goats were found to express the H19 gene (Plate. 2,3,4).

Another set of 6 goats (Nos. 463, 58, 409, 472, 495 and 58) which were confirmed as non-pregnant by abdominal palpation and/or ultrasonography and also exhibiting oestrus, detected by parading a buck and overt signs of oestrus not and mated were also subjected to H19 gene expression studies. As is evident from table 1, these goats did not express the H19 gene.

The above results show that the H19 gene is expressed only in the pregnant goats and not in the non pregnant goats.
Table 1. H19 gene expression in pregnant and non-pregnant goats

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Goat identification number</th>
<th>Approximate duration of pregnancy in months</th>
<th>H19 gene expression</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>3</td>
<td>Present</td>
<td>Pregnant</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>3 ½</td>
<td>Present</td>
<td>Pregnant</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>3</td>
<td>Present</td>
<td>Pregnant</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>3</td>
<td>Present</td>
<td>Pregnant</td>
</tr>
<tr>
<td>5</td>
<td>413</td>
<td>2 ½</td>
<td>Present</td>
<td>Pregnant</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>3</td>
<td>Present</td>
<td>Pregnant</td>
</tr>
<tr>
<td>7</td>
<td>463</td>
<td>Non pregnant</td>
<td>Nil</td>
<td>Non pregnant</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>Non pregnant</td>
<td>Nil</td>
<td>Non pregnant</td>
</tr>
<tr>
<td>9</td>
<td>409</td>
<td>Non pregnant</td>
<td>Nil</td>
<td>Non pregnant</td>
</tr>
<tr>
<td>10</td>
<td>472</td>
<td>Non pregnant</td>
<td>Nil</td>
<td>Non pregnant</td>
</tr>
<tr>
<td>11</td>
<td>495</td>
<td>Non pregnant</td>
<td>Nil</td>
<td>Non pregnant</td>
</tr>
<tr>
<td>12</td>
<td>58</td>
<td>Non pregnant</td>
<td>Nil</td>
<td>Non pregnant</td>
</tr>
</tbody>
</table>
**Phase 2**

**Studies on expression of H19 gene as an index for early diagnosis of pregnancy in goats**

Goats expressing symptoms of oestrus in a flock were isolated and mated with a buck of known fertility.

Sequential blood samples were obtained from three mated goats up to day 30 starting from day 15 following natural service or mated following induced oestrus at three days interval and analysed for expression of H19 gene. The animals following mating were observed for return to oestrus or if not returned to oestrus were subjected to pregnancy confirmation by abdominal palpation and/or ultrasonography on day 60 post mating. Further, on classification of goats in pregnant and non-pregnant, their respective H19 gene expression results were tallied for an early diagnosis of pregnancy based on H19 gene expression. The results are expressed in table 2.

As is evident from the table, goat Nos. 6 and 9 were pregnant. Sequential blood samples obtained from these goats starting from day 15 post mating up to day 30 showed expression of H19 gene (Plate 5,6). However, goat No. 34 which was classified as non pregnant did not express the gene at any day of sampling from 15 to 30 (Plate 7).

The above results clearly indicate that the H19 gene is expressed as early as day 15 of pregnancy and continues to express throughout pregnancy.

Goat no 6 and 9 were confirmed pregnant by B mode real time ultrasonography and goat no 34 not confirmed by ultrasonography.
Table 2. H19 gene expression in mated goats

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Goat identification No.</th>
<th>H19 gene expression on days of sampling post mating</th>
<th>Status of goat by H19 gene expression</th>
<th>Status of goat by abdominal palpation/ultrasonography</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>+ + + + + +</td>
<td>Express</td>
<td>Pregnant</td>
<td>Pregnant</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>+ + + + + +</td>
<td>Express</td>
<td>Pregnant</td>
<td>Pregnant</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>- - - - - -</td>
<td>Not express</td>
<td>Non pregnant</td>
<td>Non pregnant</td>
</tr>
</tbody>
</table>
5. DISCUSSION

Early detection of pregnancy aids in culling or rebreeding of barren does, and provides a valuable tool for controlled breeding programs. Inability to detect early pregnancy can result in economic losses in milk and kid production due to longer kidding intervals. In larger domestic animals such as the cow and horse, palpation of the genital tract per rectum facilitates the early diagnosis of pregnancy which is not possible in the smaller farm animals.

Many methods have been described to diagnose pregnancy in goats. These include ultrasonography (A-scan Watt et al., 1984), Doppler ultrasonic (Trapp and Slyter, 1983), Real time B-scope ultrasonics (Davey, 1986; Haibel, 1990; Bretzlaff et al., 1993), Estrone sulfate (Tsang, 1978; Refstal et al., 1991), progestrone (Murray and Newstead, 1988), radiography (Barker and Cawley, 1967), rectal-abdominal palpation (Ott et al., 1981), vaginal biopsy (Richardson, 1972a), abdominal palpation and ballotment (Pratt and Hopkins, 1975), palpation of the uterus via laparotomy (Smith, 1980) and pregnancy specific antigen (Ruder et al., 1988).

Ever since, H19 gene was first identified in the mouse as a foetal-specific mRNA expressed under the control of raf, a transacting locus gene that determines the adult basal level of α-fetoprotein (AFP) in the liver (Pachnis et al., 1988), interest to identify this gene in the embryonic tissues took a momentum. However, expression of H19 in the extra embryonic membranes was detected only after the ovine conceptus began attachment to the endometrium and the embryo itself had undergone early organogenesis (Lee et al., 2002) that may be regarded as the first step in implantation.

Establishment of pregnancy involves maternal recognition of pregnancy and implantation which is a physiological process whereby the conceptus signals, its presence to the maternal system and prolongs lifespan of the corpus luteum (CL). Progesterone from the corpus luteum acts on the uterus to stimulate and maintain uterine functions that are permissive to early embryonic development, implantation, placentation and successful fetal and
placental development to term. Maintenance of pregnancy requires reciprocal interactions between the conceptus and endometrium. Endometrial gland morphogenesis during pregnancy allows for the endometrium to increase output of secretory proteins that are transported to the fetus by specialized areas of the placenta. Histotrophic nutrition from the endometrium is the first available nutrition for the developing conceptus and appears to be essential for conceptus survival and growth throughout pregnancy in domestic animals (Spencer and Bazer, 2004).

Implantation is a critical step in the progress of pregnancy, during which the conceptus acquires a fixed position within the uterine lumen, and leads to the establishment of the placental structures. In ruminants, the implantation process is characterized by a long pre-attachment period lasting 2-3 weeks during which the conceptus elongates considerably, an apposition stage when cellular contacts are established between the trophoblast and the uterine epithelium, and an adhesion stage which ends the process and gives rise to the cellular structure of an epithelio-chorial placenta. Trophoblast apposition begins in the vicinity of the embryo by day 15, 18 and 19 in sheep, goats and cows, respectively. The trophoblast cells surrounding the embryo show morphological and functional changes.

As the implantation process spreads towards the extremity of the elongated conceptus, these cellular transformations progressively affect the whole trophoblast. Apposition is completed by a close adhesion between the interdigitating uterine microvilli and the trophoblastic plasma membrane. By this stage, trophoblastic binucleate cells migrate through the trophoblast monolayer and fuse with individual uterine cells to form a syncytiotial tissue. During this process placental lactogen hormones and pregnancy serum proteins (PSP) produced by the binucleate cells are transported to the endometrial tissues and then to the maternal blood circulation (Guillomot, 1995).

In a preliminary study in the present laboratory (Anonymous., 2011) using maternal blood, it was observed that H19 gene is expressed in maternal blood circulation in pregnant goats but not in the non pregnant
goats. This prompted to further investigate the expression of this gene as an index for early diagnosis of pregnancy in goats, since the gene has been reported to regulate the invasion, migration and angiogenesis during early pregnancy.

The present study was conducted in two phases. In the first phase, expression of H19 gene was studied in known pregnant and non-pregnant goats and in the second phase, the expression of H19 gene was studied as an index for early diagnosis of pregnancy in goats. For the purpose, sequential blood samples were obtained from three mated goats up to day 30 starting from day 15 following natural service or mated following induced oestrus at three days interval and analysed for expression of H19 gene.

In the first phase of the study, H19 gene was expressed only in the pregnant goats and not in the non pregnant goats, thus confirming its association with pregnancy. In the attempt to study the expression of H19 gene as an index for early diagnosis of pregnancy in goats, it was observed that H19 gene was expressed as early as day 15 of pregnancy and continued to express throughout pregnancy. Thus, early pregnancy at 15 days could be diagnosed by H19 gene expression in goats.

In an earlier study Lee et al (2002) analyze expression profile H19 gene by taking embroyonic tissues from fetal and postnatal tissue in sheep. During embryonic development, H19 mRNA was not detected until day 17 of gestation. At day 17, H19 was more abundant in the yolk sac and the allantois than in the trophoblast, which constitutes the bulk of the conceptus at this stage. In the day 17 trophoblast, only the mesoderm and not the trophectoderm expressed H19. The identity of the two trophoblast cell layers was verified with the detection of IFN-τ mRNA only in the trophectoderm but not the mesoderm. Of all the extraembryonic membranes of the conceptus at day 17, the yolk sac expressed the highest levels of H19. Generally, derivatives of the mesoderm and endoderm, such as liver, heart, gut, muscle and kidney expressed high levels of H19, whereas those of neuroectodermal origin, such as the brain, did not.
However, no effort were made to detect the H19 gene expression from maternal or fetal blood samples after pregnancy. Thus, It can be concluded that the H19 gene is expressed as early as day 15 post mating in the blood of goat and is expressed 30 days of pregnancy. It is not expressed in non pregnant goats at any stage of cycle. The expression of H19 gene can be used for diagnosis of pregnancy in goat as early as day 15 which correlates with the period of implantation of embryo. This early detection of H19 gene in goat pregnancy may act as indicator of successful implantation of embryo in goat in MOET and IVF-ET programs.
6. SUMMARY, CONCLUSION AND SUGGESTIONS
FOR FURTHER WORK

6.1. Summary

The present work was conducted with the objectives (i) to study the expression of H19 gene in known pregnant and non-pregnant goats, and (ii) to study the expression of H19 gene as an index for early diagnosis of pregnancy in goats.

In the first phase, expression of H19 gene was studied in known pregnant and non-pregnant goats and in the second phase, the expression of H19 gene was studied as an index for early diagnosis of pregnancy in goats. For the purpose, sequential blood samples were obtained from three mated goats up to day 30 starting from day 15 following natural service or mated following induced estrus at three days interval and analyzed for expression of H19 gene.

In the first phase of the study, H19 gene was expressed only in the pregnant goats and not in the non pregnant goats. In the attempt to study the expression of H19 gene as an index for early diagnosis of pregnancy in goats, it was observed that H19 gene was expressed as early as day 15 of pregnancy and continued to express 30 days of pregnancy. Thus, early pregnancy at 15 days could be diagnosed by H19 gene expression in goats.
6.2. Conclusion

- H19 gene is observed as early as day 15 post mating in goat and maintained 30 days of pregnancy in goat. The gene is not expressed in any stage of cycle in non pregnant goats.

- The expression of H19 gene can be used for diagnosis of pregnancy in goat as early as day 15 which correlates with the period of implantation of embryo.

- This early detection of H19 gene in goat pregnancy may act as indicator of successful implantation of embryo in goat, MOET and IVF program.

- H19 is more likely to be act molecular marker for pregnant and non pregnant goat.
6.3. Suggestions for Further Work

1. H19 expression can be correlated with early embryonic mortality in farm animal.

2. The expression profile of H19 gene using Real Time PCR should be standardize at different stage of pregnancy.

3. The study can be extended in other farm animals to know the possible role of H19 gene as an indicator of successful implantation of embryo for detection of early embryonic mortality.

4. The expression profile of H19 gene along with its closely linked gene like IGF-2 and its receptor can be studied during early fetal growth and subsequent development.
REFERENCES


VITA

Dr. Sunil Kumar was born on 15 April 1981 in Village Vanvaripur Post. Marthara Bhagwan Das, Distt. Etah (Uttar Pradesh). He passed his Higher Secondary from Government Inter College Etah with Second Division. He earned his B.V.Sc. and A.H. Degree from College of Veterinary Sciences and Animal Husbandry Mathura, (DUVASU, Uttar Pradesh) in 2010. In the same year he was selected for M.V.Sc & A.H. (Animal Biotechnology) Post Graduate degree program in Animal Biotechnology Center with Deptt. of Biotechnology, Govt. of India, New Delhi, Junior Research Fellowship at Madhya Pradesh Pashu Chikitsa Vigyan Vishwa Vidyalaya, Jabalpur (M.P).

Sunil Kumar Rajpoot
Awas Vikas Colony
ETAH (U.P.)
Email: vetsunilrajpoot@gmail.com
Contact: 08430703418