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An overview of pregnancy diagnosis in small ruminants

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ABSTRACT

Accurate diagnosis of early pregnancy is a key factor for successful reproduction management in sheep and goat farm. Methods of pregnancy diagnosis depending on visualization of the conceptus or determination of its secretory products in the maternal blood or in the milk are the most accurate and specific methods for pregnancy. The use of determination of PAG concentration can help for pregnancy diagnosis and for new investigations on embryonic or fetal mortalities. The impetus is to develop reliable and practical on-farm/ranch tests for early pregnancy based on the presence of hormones or pregnancy-associated proteins. Non-return to estrus is still the easiest and cheapest method applicable at field and farm level. Real-time B-mode ultrasonic scanning of the uterus in goats and sheep appears to offer an accurate, rapid, safe and practical means for diagnosing pregnancy (from day 19), determination of fetal numbers, as well as for the identification of sex and prediction of gestational age and calving date in field conditions. The optimum time for using transabdominal or transrectal ultrasonography in small ruminants ranges from 19 to 100 days of gestation. Other techniques like laparoscopy, laparotomy, vaginal cytology and radiography although reliable are limited to the laboratory because of infrastructure and cost involvement. In many developing countries, farmers need to be educated on getting their animals checked for pregnancy at an early date as it was found that the earlier the pregnancy diagnosis performed the better the production and reproduction.

Key words: Accuracy, Cost, Goat, PAG, Practical, Pregnancy Diagnosis, Radiography, Sheep, Ultrasonography

The socio-economic importance of small ruminants is evident by the sharp increase in their numbers and contributions. They contribute milk, meat, fibre, skins and manure to the subsistence of small holders, landless rural poor and play an important role in capital storage, income and employment generation and house hold nutrition. Like other species, productive potential of small ruminants is greatly affected by reproductive management (Das *et al.* 2011). An early diagnosis of pregnancy is needed for improving reproductive efficiency and maintaining cost-effective production. Early detection of pregnancy is of considerable economic value to a sheep and goat farm (Meena *et al.* 2003, Kharche 2005, Kharche and Goel 2005). Predictions of the number of fetuses would allow appropriate nutritional management of females in late gestation that will prevent pregnancy toxemia (Ford 1983), minimize prelambing feeding costs, optimize birth weight, weaning weight and survivability of lambs and reduce incidence of dystocia (Gearhart *et al.* 1988). In addition, accurate information on the stage of gestation would be useful to dry off lactating females at adequate period and to monitor the females near term (Doize *et al.* 1997). A highly valued zygote or embryo when transferred to a less valued surrogate mother (recipient) need to be closely monitored and the early detection of conception helps in repeated use of barren females. Proper management of pregnant animals also prevents embryonic losses. There are many methods of pregnancy diagnosis currently in use. The method applied should be safe to both offspring and dam and needs to be low cost and easily applied. Various methods are used to diagnose pregnancy in sheep and goats. Some of them have some limitations to their wide scale use. They are classified into 3 categories: visual methods, clinical methods and laboratory tests (Kharche and Triveni Dutt 2003, Purohit 2010). A review on use of these various techniques in small ruminants is presented.

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General physiology of reproduction in small ruminants

To track the stages of pregnancy in small ruminants and accurately predict the due date, it is important to know their estrus cycle. A comparison of reproductive characteristics of sheep and goats are presented in Table 1. A goat in estrus is more obvious than a sheep.

Owing to limitation of structural anatomy of genital organs to examine uterus and ovaries per rectum, there was a need to develop alternative method for routine pregnancy diagnosis in small ruminants.

Table 1. Reproductive characteristics of sheep and goat

Reproductive characteristics	Sheep	Goat
[1,2]Length of estrus cycle	17 days (14–19)	21 days (18–22)
[2]Type of estrus cycle	Seasonal poly oestrus	Seasonal poly oestrus
[1,2]Duration of estrus	30 h (15–45)	36 h (24–72)
[1,2]Optimal breeding time after onset of estrus	Towards end of estrus, e.g 18–24 h	At acceptance of buck (24 h) and again in 12 h
[1]Ovulation rate	Breed dependent-multiple common	As for sheep – most breeds prolific
[1]Behavioural estrus	Anorexia; vulvar swelling; small amounts of mucus; follow ram; more subtle than goats	Restless; wag tail; vocalize; swollen vulva; clear vagina mucus; follow buck; very demonstrative
[1]Pregnancy progesterone	Secreted by corpus luteum but mostly placenta after day 75	Secreted only by corpus luteum
[1,2]Gestation length	144–151 days	147–155 days
[1]Cervical anatomy	Complex 7 rings and offset	3 to 6 rings and aligned

[1]Menzies (2008); [2]Kouamo and Kharche (2014).

Visual methods

Non return to oestrus: When a mated animal does not return to oestrus, the usual assumption is that the animal is pregnant and hence has not returned to oestrus. This happens because during pregnancy, conceptus inhibits regression of corpus luteum and thus prevents the animal from returning to oestrus. However, many a times the animal does not return to oestrus because of non regression of CL due to reasons other than pregnancy. Moreover, in the seasonally breeding species the animal may not return to oestrus (when mating is done during the end of the breeding season) because the season is over. Anestrus, and the rare occurrence of gestational oestrus in sheep and goats can affect reliability of non return to oestrus as a method of pregnancy diagnosis. Likewise, oestrus detection methods used in cattle (Kharche and Srivastava 2001) need to be properly designed for sheep and goat so as to make efficient use of non-return to oestrus as a method of pregnancy diagnosis in these species (Purohit 2010).

Other visual signs: Besides the non-return to oestrus other visual signs of pregnancy appearing in late pregnancy include increase in the size of abdomen, development of udder, slight vaginal discharge and movements of the fetus visible externally (Kharche 2008, Goel and Kharche 2009, Purohit 2010). However, accuracy of these visual diagnostic symptoms is always low and a clinician must use them as a supplement to clinical diagnosis.

Clinical methods

Clinical methods of pregnancy diagnosis, available in the various domestic farm and pet animal species, are: rectal palpation, abdominal ballottement, radiography, ultrasonography and laparoscopy.

Rectal palpation: Transrectal palpation is the oldest and most widely used method for early pregnancy diagnosis in cattle (Cowie 1948, Shrivastava and Kharche 2002). It is the easiest, cheapest and fastest method of pregnancy diagnosis with little or nil harm to the animal and its fetus when performed carefully. Rectal palpation in small ruminants is of little value due to the size of the pelvis (Wani *et al*. 1998). In sheep and goats, rectal abdominal palpation (by using a glass rod placed in the rectum to lift the uterus which is palpated through abdomen) is suggested (Chauhan and Waziri 1991). Similarly bimanual palpation for pregnancy diagnosis (palpation of uterus through fingers in the rectum and lifting the abdomen) was reported for small ruminants (Kutty 1999). The fetus is palpated through the abdominal wall by moving the stick side-to-side while applying slight upward pressure; the method is reliable after 50 days of gestation. Movements of the fetuses can often be palpated between 3½ and 4 months. The right flank is the most promising area to feel movement, as the rumen takes a lot of room on the left. Although this technique is simple, cheap and quick (150 ewes can be examined per hour), it had a low accuracy in diagnosing multiple fetuses, often invasive and was more hazardous with respect to rectal injury (Tyrrell and Plant 1979) and abortion (Ishwar 1995).

Abdominal ballottement and abdominal palpation: Palpation of fetuses through the abdomen is possible in sheep and goat only beyond 4 months of pregnancy by lifting the abdomen held between both hands and location of bony fetal structures. However, sometimes bezoars in the rumen may confuse with pregnancy (Kharche and Goel 2008, Purohit, 2010)**.**

Radiography: Radiography is used for pregnancy diagnosis to a limited extent in the small ruminants (sheep and goat). Ford *et al*. (1963) examined 322 ewes by radiography and reported 100 and 90 % accuracy for diagnosing pregnancy and determination of the fetal number, respectively, after 70 days of gestation. Grace *et al.* (1989) reported 94 to 100% accuracy of radiography for determining fetal numbers in 13 sheep flocks. Besides the accuracy, the technique is quick; 400 to 600 ewes can be tested per day under farm conditions. Mostly, a single radiograph taken with the animal in lateral recumbency is sufficient however, sometimes a dorsal or a dorso ventral view may be required. In sheep and goat, fetuses are visible by day 70 of gestation (Noakes 1999) with a high accuracy. The overall accuracy of the method in detecting pregnancy increases with advancing gestation: 52% between 66 and

95 days to 100% after 96 days (Richardson 1972). The accuracy of determining fetal numbers approaches 87% only between day 91 and 110 (Ardran and Brown 1964). Therefore, radiography is suggested to be done only after day 90 in sheep and goat. Moreover, the high cost and the hazards of exposure to growing fetuses to X rays limit the use of radiography as a routine procedure, and warrant its use in specialized cases (Purohit 2010).

Ultrasonography: In the past 30 years, three types of ultrasonographic systems were used for pregnancy diagnosis in the small ruminants.

A-mode ultrasound (Amplitude-depth or echo-pulse): In this system, the transducer emits ultrasound waves which penetrate tissues under the skin and are reflected when meet high acoustic impedance interfaces (pregnant uterus or fluid-filled structures). The transducer receives the reflected echoes and converts it into peaks on oscilliscope with horizontal scale representing the depth of the reflecting structure or into audible signal. Meredith and Madani (1980) used reflection of ultrasound at depth 9 cm or greater as a positive sign of pregnancy in ewe and reported 96 % sensitivity and 87.5 % specificity in the period from 61 to 151 days after mating. However, by the same approach, lower sensitivity (86.7 %) and specificity (69 %) was reported in the ewe lambs at days 73 to 103 post mating (Madel 1983). By using echo-pulse detectors, the accuracy for detecting pregnant ewes averaged 91% from days 69 to 112 of gestation (Trapp and Slyter 1983). However, Watt *et al.* (1984) reported 97 % accuracy for diagnosing pregnancy from day 51 of gestation to lambing. A-mode ultrasound is a quick, convenient and simple technique, but it cannot predict the fetal number and the viability of the fetus.

Oppler ultrasound: Doppler devices utilize the Doppler shift principle to detect the fetal heartbeats and flow of blood in uterine and fetal vessels. The intrarectal Doppler technique could be used for diagnosing pregnancy at the beginning of the second trimester with an accuracy of 90% or better (Lindahl 1971). The accuracy of intra rectal Doppler transducer for diagnosing pregnancy and non-pregnancy was 82 and 91 %, respectively, from days 41 to 60 of gestation (Deas 1977). After day 71, the accuracy for diagnosing pregnancy and non pregnancy ranged between 85 and 94 %, respectively (Watt *et al.* 1984). In contrast, Trapp and Slyter (1983) reported 68 and 84 % accuracy for diagnosing pregnancy and non-pregnancy from days 60 to 96 of gestation. The use of an external Doppler transducer gave almost 100% accuracy for diagnosing pregnancy after day 111 of gestation (Watt *et al*. 1984).

Concerning the predictions of fetal numbers, the external Doppler technique, when used by skilled operator gave 83 and 93 % accuracy for diagnosing single and multiple fetuses at days 80 to 95 of gestation, respectively (Fukui *et al*. 1986). However, Fukui *et al*. (1984) reported 74 and 89% accuracy for ewes carrying singles and multiples, respectively, from days 60 to 120 of gestation. Doppler devices have not been used successfully for estimating ovine gestational age (Russel and Goddard 1995).

Real-time, B-mode ultrasonography: Real-time B-mode ultrasonic scanning of the uterus in small ruminants appears to offer an accurate, rapid, safe and practical means for diagnosing pregnancy, determination of fetal numbers and estimation of gestational age.

Diagnosis of pregnancy

Goddard (1995) described methods of evaluation of pregnancies using ultrasonography for various species. The cotyledons are visible between day 30–40 and fetal extremity/bone by day 57–60 in cattle, day 70 in sheep and day 50 in goat. A trans rectal probe of 5.0 to 7.0 MHz frequency (usually a prostatic probe used in human beings) is required to diagnose pregnancy between days 25–30 in sheep and goats (and this can be used to study non pregnant uterus and ovaries) but under field conditions a transabdominal probe (linear or sector 3.5 to 5.0 MHz frequency) is generally used and this can diagnose pregnancy earliest at day 40–50 in sheep and goats with reasonable accuracy as the fluid and cotyledons are easily visible sonographically at this time (Duggal *et al*. 2001, Suguna *et al.* 2008, Goel *et al.* 2009).

By using transrectal ultrasonography (7.5 MHz), embryonic vesicle of the pregnant Manchega dairy ewe was identified at day 12 after mating, while the first visualization of the embryo was at day 19 (Gonzalez *et al.* 1998) or day 20 (Schrick and Inskeep 1993). Karen *et al.* (2002) reported that the age of the Awassi × Merino ewes has an effect on the accuracy of transrectal ultrasonography for detection of pregnancy. In addition, fasting Awassi × Merino ewes for 12 h prior to scanning and lifting up their abdomen while conducting the scanning significantly improve the accuracy of the transrectal ultrasonography for detecting early pregnancy.

By using transabdominal approach, pregnancy was first verified at day 25 (Gearhart *et al.* 1988) or day 30 after breeding (Bretzlaff *et al.* 1993). The sensitivity and specificity of the technique were high after day 29 (Taverne *et al.* 1985) reaching approximately 100% from days 46 to 106 of gestation (Gearhart *et al.* 1988). However, Logue *et al.* (1987) reported a lower specificity at days less than 40 to 100 after mating. Hussein (2008) reported that the B-mode transrectal (TR) probe enabled more reliable and early recognition of foetal fluid (5 days) and heart beats (4 days) than the B-mode transabdominal (TA) probe.

Kouamo *and* Sawadogo (2011c) conducted a study on pregnancy diagnosis after artificial insemination on sahelian goats. They reported a pregnancy rate of 56.43 % to day 40 and 72.27% to day 60 by using transabdominal approach with a sectoral probe of 3.5MHz, an axial resolution of 1mm, a lateral resolution of 2 mm and a depth of 22 cm. The sensitivity was higher than specificity. The technique was not very accurate before day 21 for pregnancy diagnosis (Kouamo *et al*. 2011b). In sub-Saharan Africa, the method of pregnancy diagnosis by non return to oestrus remains the most applicable at farm level. The stockbreeders must

be educaed on early detection of pregnancy and the use of real-time B ultrasonography.

Determination of the fetal number

By using transrectal ultrasonography (7.5 MHz), single and multiple pregnancies in sheep were accurately (15 of 17 ewes) detected at day 25 (Schrick and Inskeep 1993). However, the accuracy of a 5 MHz transrectal ultrasonography for detecting ewes carrying 2 fetuses or more was disappointing (Gearhart *et al.* 1988). By using transabdominal ultrasonography, the accuracy of experienced operator for determination both single-and multiple-bearing ewes was 99 % from days 46 to 93 of gestation (White *et al.* 1984). A similar accuracy for ewes carrying single fetus was reported by Gearhart *et al.* (1988). Goel and Agrawal (1992) reported that it is difficult to differentiate between twins and triplets or quadruplets at any stage of gestation while Karen *et al.* (2004) reported that the accuracy of ultrasound in detecting ewes carrying 2 foetuses or more was disappointing. McGahan and Coats (1996) reported that multiple pregnancies should be reported in instances where multiple embryos are detected.

Estimation of gestational age

When the date of mating is unknown, monitoring fetal development allows estimation of gestational age.

Embryonic vesicle: Gonzalez *et al.* (1998) measured the ovine embryonic vesicle from days 12 to 29 of gestation by using 7.5 MHz transrectal ultrasonography and found a close correlation (r = 0.76) with the gestational age.

Crown-rump length: By using transrectal ultrasonography (7.5 MHz), Schrick and Inskeep (1993) measured the crown-rump length of the ovine fetus from days 20 to 40 of gestation and described the relationship between the crown-rump length (x) and the gestational age (y) by the following equation, $Y=14.05 +1.16x - 0.012x^2$. By using the same approach, Gonzalez *et al.* (1998) reported a high (r = 0.94) correlation between the crown-rump length and the gestational age from days 19 to 48 of gestation. Hussein (2008) working on Egyptian Baladi goats reported a relationship between gestation age and crown-rump length (CRL) and bi-parietal diameter (BPD) from days 40 to 109 of gestation. The TA convex probe was used from days 40–89 to measure CRL and days 40–109 to measure BPD.

Fetal head diameters: Fetal head diameters including the biparietal diameter, the occipito-nasal length and the diameter of the orbit were used to predict the stage of gestation in sheep.

Regarding the biparietal diameter (BPD), Gonzalez *et al.* (1998) used the transrectal ultrasonography to measure the BPD of Manchega sheep from days 32 to 90 and found a high correlation (r = 0.96) between the measured diameters and the gestational age. Similar correlation was found by using transabdominal approach in Suffolk and Finn sheep from days 40 to 95 (Haibel and Perkins 1989), in Booroola × South Australian Merino sheep from days 49 to 109 (Sergeev *et al*. 1990) and in Swedish peltsheep from 10 weeks before lambing to birth (Aiumlamai *et al*. 1992). Kelly and Newnham (1989) found the occipito-nasal length to be more accurate than BPD, showing a linear increase till day 80. However, Sergeev *et al.* (1990) reported that the occipito-nasal length was more difficult to be measured than BPD and had the same accuracy for predicting fetal age. Gonzalez *et al.* (1998) found a high correlation (r = 0.95) between the fetal occipito-nasal length and the gestational age from days 38 to 91 of gestation.

Regarding the diameter of the fetal orbit, Gonzalez *et al.* (1998) reported that the ovine fetal orbit increased in diameter from 2 mm at day 36 to 17 mm at day 90 of gestation and it gave a high correlation (r = 0.92) with the fetal age.

Thoracic diameter: Ultrasonographic measurements of the ovine fetal thoracic diameter showed high correlation with the fetal age from days 49 to 109 (Sergeev *et al.* 1990) and from days 23 to 90 of gestation (Gonzalez *et al*. 1998).

Fetal heart rate: The fetal heart beat can be seen between day 24–30 and the fetus itself between days 25–30 in most species. By using 7.5 MHz transrectal ultrasonography, the rhythmic pulsations within the ovine embryonic vesicle were first detected at day 18 or 19 after mating (Schrick and Inskeep 1993), while by using 5 MHz transrectal ultrasonography, they were first observed from days 21–23 after mating (Garcia *et al.* 1993). Aiumlamai *et al.* (1992) measured the ovine fetal heart rate during the second half of pregnancy by using transabdominal ultrasonography and reported that the fetal heart rate reached the plateau at 7 weeks before lambing (167 ± 1.5 bpm) then decreased at 3 weeks before lambing (139.0 ± 15.7 bpm) and reached 117.0 ± 9.2 bpm at birth. In addition, a significant correlation was found between fetal heart rate and gestational age. Hussein (2008) reported that the TR observation of heat beats is recommended as conclusive evidence of the presence of a live foetus.

Placentome size: Placentomes could be detected by transrectal ultrasonography (5 MHz) at day 30 (Buckrell *et al*. 1986) and at day 32 of gestation (Doize *et al*. 1997). At this period the placentomes appeared as echogenic areas on the surface of endometrium. At day 42, the ovine placentomes presented cup-shaped forms and reached the maximum size by day 74 (Doize *et al*. 1997). There was a poor correlation between placentome size and ovine gestational age due to great variation in the size of placentome in the same observations (Gonzalez *et al.* 1998). In contrast, Kelly *et al.* (1987) found a significant quadratic relationship between ultrasonographic cotyledon diameter and square root transformation of day of pregnancy.

Other fetal structures: There was a high correlation (r = 0.96) between the width of 3 ovine fetal coccygeal vertebrea and gestational age. At the same time, somewhat lower correlation was found for umbilical cord diameter (r = 0.72) and fetal femur length (r = 0.78) (Gonzalez *et al.* 1998).

Determination of fetal sex

Depending on the location of the genital tubercle of the

ovine fetus, the accuracy of the transrectal ultrasonography (5 MHz) for detecting male and female fetuses was 100 and 76%, respectively, from days 60 to 69 of gestation (Coubrough and Castell 1998). Hussein (2008) reported that identification of genital tubercle in male fetuses is possible from day 40 onwards. In a previous study using goats of Anglo-Nubian breed, Santos *et al.* (2005) concluded that foetal sex determination by ultrasonography in goats should not be performed after day 100 of pregnancy, especially in multiple pregnancies. However, after birth, one case was misdiagnosed by ultrasound i.e. 83.3 and 68.4% for single and multiple births were compared to the total sexing by ultrasound (83.3 and 70.2% of single and multiple births).

Laparoscopy

Laparoscopy can be used as a method of pregnancy diagnosis by directly visualizing the genitalia in animals however, the invasive nature of the technique, the high cost of equipment and clinic required, and the availability of non invasive techniques limit the use of this technique as a means of pregnancy diagnosis in goat and sheep (Kharche and Goel 2009, Purohit 2010).

Laboratory test

The various laboratory tests developed for pregnancy diagnosis in domestic animals are indirect methods of pregnancy evaluation, and utilize qualitative or quantitative measures of reproductive hormones at specific stages after AI or mating, or detect conceptus specific substances in maternal body parts or body fluids as indirect indicators of the presence of a viable pregnancy (Purohit 2010).

Progesterone

Measurement of blood progesterone concentration is a reliable indicator of the functional corpus luteum. Concentration of plasma progesterone samples was determined in ewes at day 18 post-breeding by using enzyme immunoassay (EIA) and radioimmunoassay (RIA). The accuracy of both types of assays for detecting pregnancy was high, while it was low for diagnosing non-pregnancy (Gvozdic and Ivkov 1994). On the other hand, 100 % accuracy for detecting non pregnant ewes was achieved by using EIA at day 16 (McPhee and Tiberghien 1987) and day 21 after mating (Zarkawi 1997) or by using RIA at days 17 to 18 (Zarkawi *et al*. 1999). Early embryonic death, uterine and/ or ovarian pathology may be the source of the false positive cases. Commercially available ELISA, plasma or milk progesterone assay kits have not become popular due to their high cost and a low specificity. Non pregnant goat and sheep not returning to oestrus and pregnant goat and sheep in which embryonic death occurs at a later time can both give false results.

Likewise sheep and goats assay of plasma or milk progesterone is not very accurate for diagnosis of pregnancy (Zarkawi 1997, Goel *et al.* 2009).

Estrone sulphate

The estrone sulphate is produced by the feto maternal axis or the conceptus and therefore its presence in urine, milk, feces or blood is an indicator of pregnancy. The detection of estrogens depends on the availability of suitable laboratory and availability of commercial assay kits. Laboratories evaluating concentrations of estrogens in urine or serum usually are equipped with radioimmunoassay, enzyme immuno-assay or other more precise and specific diagnostic modalities for assay of steroids in urine, serum, feces (Bamberg *et al.* 1984) or other body fluids. Evaluation of steroids like estrogen from feces is especially helpful for zoo and feral species where faeces are the most easily collected specimens (Bamberg *et al.* 1991, Hermann *et al.* 2005). The presence of a viable feto-placental unit is accompanied by an increase in estrone sulphate concentrations in the peripheral plasma of ewes. Estrone sulphate was detectable around day 70 of gestation with value ranging between 0.1 to 0.7 ng/ml, then its level increased steadily till 2 days before parturition when an upsurge was seen (15–50 ng/ml) (Tsang 1978). At day 85 of gestation, there was a significant difference in the level of estrone sulphate between pregnant and non-pregnant ewes. However, due to considerable variation of the hormone levels between individuals, the accuracy for detection of non-pregnancy was only 44 % whilst for detection of pregnancy was 87.9 % using the cut-off value of 0.1 ng/ml (Worsfold *et al.* 1986). On the contrary, Illera *et al*. (2000) reported that the EIA test for the measurements of serum estrone sulphate concentrations gave an optimal accuracy for pregnancy diagnosis between days 30 to 35 of gestation.

Regarding the fetal number, the concentration of serum estrone sulphate was significantly higher in ewes carrying multiple than those carrying single fetus from days 80 to 124 of gestation (Illera *et al*. 2000). However, the determination of estrone sulphate concentrations in ovine blood might not be reliable for prediction of fetal numbers due to the high variation between individuals (Worsfold *et al*. 1986).

Ovine chorionic somatommamotrophin (ovCS) or ovine placental lactogen (ovPL) and caprine placental lactogen (caPL): ovPL and caPl is purified and commercialize by many lab. Radioimmunoassay of ovPL achieved 97 and 100 % accuracy for diagnosing pregnant and non- pregnant ewes at day 64 of gestation, respectively (Robertson *et al.* 1980).

Pregnancy proteins

Ovine PAG family: As early as in 1988, the identification of placental antigens immunologically related to bovine PAG/PSPB in the peripheral circulation of pregnant ewes (Ruder *et al*. 1988) has encouraged their isolation and characterization in this species. Isolated molecules took the following names: ovine PAG (ovPAG) (Zoli *et al.* 1995), ovine PSPB (oPSPB) (Willard *et al.* 1995) and SBU-3 antigen (Atkinson *et al.* 1993). The first purified and

characterized ovine PAG (designated ovPAG 1) was initially identified as a molecule closely related to the boPAG–1, containing 382 amino acids. Molecular cloning studies have shown that ovPAG–1, like boPAG–1, belongs to the aspartic proteinase family showing 73% amino acid sequence identity with this protein (Xie *et al.* 1991). Two years later, a cDNA coding for an additional member of PAG family (ovPAG–2) was identified in ovine placental library prepared from day 13 whole conceptus (Nagel *et al.* 1993). More recently, 9 additional cDNA coding for distinct PAG (ovPAG–3 to ovPAG–11) were identified in ovine placenta at different gestational periods, confirming the multiplicity and temporal expression of PAG molecules in ruminant placenta (Green *et al*. 2000).

As previously described for boPAG family, ovPAG–2 is expressed in both mononucleate and binucleate cells throughout the trophectoderm whereas a large number of ovPAG molecules (ovPAG–1 and ovPAG–3 to –9) are predominantly expressed in binucleate trophectoderm cells (Green *et al*. 2000). Several ovPAG have been purified from conditioned media from 100 day-ovine placental explants (Xie *et al.* 1997), as well as from cotyledonary tissue collected between day 60 to 100 (El Amiri *et al.* 2004) and after day 100 of pregnancy (El Amiri *et al.* 2003). By use of a series of chromatography procedures, 15 ovPAG having molecular masses ranging from 55 to 66 kDa (ovPAG55kDa to ovPAG66kDa) could be isolated and characterized (El Amiri *et al*. 2004). Another subset of PAG was identified in extracts of sheep placenta by Atkinson *et al*. (1993) who used the monoclonal antibody against SBU-3 developed by Gogolin-Ewens *et al.* (1986). The 3 proteins affinity-purified with the SBU-3 antibody had molecular weights of 57, 62 and 69 kDa, showing partial amino acid sequence homology to the ovPAG–1, boPAG–1 and rabbit pepsinogen F. The analysis of glycan (or carbohydrate) content of SBU-3/ovPAG were consistent with the major chains being sialylated and having multiple antennary N-linked chains (17.83% of the relative molecular mass of ovPAG/SBU3) (Atkinson *et al.* 1993). In these molecules, the large amounts of lateral chain sugars have been shown to be made up of N-acetyl glucosamine (5.26%), N-acetyl neuraminic acid (4.25%), N-acetyl galactosamine (3.62%), galactose (1.94%) and mannose (1.81%). As described by Xie *et al.* (1997) potential sites for glycosylation range from 2 (ovPAG–2) to 7 (ovPAG-8) in ovine species. However, biological implications related to glycan contents of PAG could not be demonstrated experimentally.

Pregnancy diagnosis in ewes: Pregnancy diagnosis by PAG RIA can be performed in both plasma (Vandaele *et al.* 2005), and milk samples (El Amiri *et al*. 2003). The measurement of peripheral concentrations of PAG is a reliable method for early pregnancy diagnosis at farm conditions, having equivalent results than progesterone determination and even ultrasound (Karen *et al.* 2006). In this aspect, the determination of plasmatic concentrations of PAG/PSPB molecules in ewes can give useful information to develop appropriate feeding strategies for pregnant females and to insure requirements of the mother and fetuses growing to avoid metabolic disorders associated to pregnancy (El Amiri *et al.* 2003). PAG can be detected in sheep maternal blood at the third to fourth week after breeding (Willard *et al*. 1995). In a more recent investigation, by the use of 2 ovine-based PAG RIA systems (RIA-780 and RIA-805), we demonstrated that from day 18 onward, all non pregnant ewes had undetectable or lower ovPAG concentrations than those described in pregnant females (El Amiri *et al*. 2007). PAG profiles in pregnant ewes (Ledezma-Torres *et al*. 2006) are quite different than those obtained in cattle (Zoli *et al*. 1992). In Churra and Merino ewes, for example, after a first period of high concentrations around day 60 of gestation, the concentrations decrease until day 90 and increase again to remain elevated and stable until parturition. After parturition, the rate of decline in PAG concentrations is faster in ewes (4 weeks) than in cows (about 14 weeks) (Ranilla *et al.* 1994). Ovine PSPB might be a useful marker of placental development and function and provide a reliable indicator of fetal distress and adverse pregnancy outcome. Between days 50 and 100 of gestation, ovPSPB concentrations were positively correlated with placental weight at term (Sousa *et al*. 2006).

Caprine PAG family: Three different PAG molecules were isolated and partially characterized from goat placenta (Garbayo *et al*. 1998). These proteins differed in amino acid sequence and apparent molecular masses (Wooding *et al.* 2005), showing several isoforms with different isoelectric point (pI): caPAG55kDa (pI: 5.3, 5.1, 4.9), caPAG59kDa (pI: 6.2, 5.9, 5.6) and caPAG62kDa (pI: 5.1, 4.8). Caprine PAG showed high sequence homology to each other (60 to 73 % residues identical) and a high sequence identity (from 30 to 81 % between the first 27 amino acids sequenced) with proteins of the aspartic proteinase family like boPAG–1, ovPAG–1, boPAG–2, SBU-3 rabbit pepsinogen F and horse PAG (Garbayo *et al.* 1998). Garbayo *et al.* (2000) described a total of 11 cDNA coding for PAG molecules in caprine placental libraries (caPAG–1 to caPAG–11) (28); 9 of those molecules (caPAG–1, caPAG–3 to –7 and caPAG–9 to –11, named caPAG–1 group) displayed more than 80% sequence identity with each other, expressed in trophoblast binucleate cells. By contrast, caPAG–2 (as boPAG–2 and ovPAG–2) was found expressed throughout the trophectoderm during early pregnancy (days 18 and 19). Also as described in bovine species, caPAG–2 is of more ancient origin than caPAG–1 group, being more recent than caPAG–8.

Pregnancy diagnosis in caprine: The use of specific antisera (AS#706 and AS#708) allowed the discrimination between pregnant and non pregnant goats as early as 21 days after breeding (Gonzalez *et al.* 1999). However, its use in farm conditions is recommended after day 26 and 32 in plasma and milk samples, respectively (Gonzalez *et al*. 2004). As observed in cattle (Patel *et al*. 1997), both number and genotype of fetus can influence PAG concentrations over gestation. From days 21–24 and throughout gestation,

twin bearing goats have higher PAG (or PSPB) concentrations than goats bearing one fetus (Gonzalez *et al*. 2000). Sequential measurement of PAG in goats also allows for the determination of the onset of the disturbance of trophoblastic activity associated with the death of a fetus (Faye *et al*. 2004). As demonstrated by Zarrouk *et al*. (1999) in goats carrying a single fetus, PAG levels fall under the positive pregnancy threshold when the trophoblast died, while in goats carrying 2 or 3 fetuses, analysis of the profiles clearly showed marked drops in concentration that could indicate placental distress at different times (Zarrouk *et al*. 1999). Therefore, systematic application of this test in herds with a high rate of pregnancy failure could help to identify endocrinological phenomena which might be implicated in triggering these events.

Early pregnancy factor: This protein molecule was first identified in pregnant mice (Morton *et al.* 1987) and later in sheep and cattle (Nancarrow *et al*. 1981) by using the rosette inhibition bioassay which detected EPF. With this assay, EPF was detected in the serum of all mammals tested within 24 to 48 h of fertilization and disappeared within 24 to 48 h after death or removal of embryo (Morton *et al*. 1987). Early pregnancy factor is an immunosuppressant that was first reported in pregnant women, and subsequently in livestock. A chemical test for pregnancy termed early pregnancy factor (EPF) or early conception factor (ECF) was proposed and marketed in the 1990's. Chen and Zhang (1992) studied determination of the serum early pregnancy factor and its characteristics in goats. A modified rosette inhibition test with mouse spleen cells was set up. The rosette inhibition titres of pregnant mouse sera were significantly higher than that of non-pregnant mouse sera ($P<0.01$), which indicated that early pregnancy factor did exist in pregnant mouse serum. After fractionated with DEAE-cellulose and eluted with pH 7.0, 0.05 mol PBS buffer, the mean rosette inhibition titre in caprine pregnant sera was significantly higher than that of non-pregnant, oestrus, and male goats ($P>0.001$), but serum rosette inhibition titres among the goats under late 3 physiological status were observed at the same level. They concluded that early pregnancy factor also existed in pregnant serum in goats. Moreover, the early pregnancy factor activity appeared within 24 h after mating, and sustained at pregnant level until day 21 after mating. These results implied that detection of caprine early pregnancy factor in serum could be used in early diagnosis of pregnancy in goats.

Samik (2007) tried to find out a substance that was useful in early pregnancy diagnosis of Ettawa crossbred goat. Characterization of EPF protein was conducted; and concentration of EPF protein isolate was measured. Antibody against EPF was produced in male rabbit, and it was measured using ELISA indirect. The test of recognition of anti EPF antibody toward EPF protein of goat cotyledon was conducted using immunohistochemistry method. The result indicated that molecular weight of EPF protein was between 42.7 and 66.4 kDa, with the average of concentration of EPF protein in blood serum of 11.182±1.116 g/ml. EPF protein could induce anti EPF antibody. He concluded that EPF protein of goat cotyledon was recognized by anti EPF antibody, and suggested to develop as a kit of early pregnancy diagnostic for goat.

Aquariyanti *et al.* (2008) determined goat pregnancy using bovine anti-early pregnancy factor polyclonal antibody. Bovine early pregnancy factor polyclonal antibody detected pregnancy with an accuracy 94.44% and a high specificity (91.67%) from day 21 of gestation. They concluded that EPF that is from bovine cotyledon was reliable for detecting pregnancy in goat.

The possibility of performing an early pregnancy diagnosis (within 1 day after insemination) is, of course, intriguing in as much as it may be possible to resynchronize within 1 week after insemination (Lucy *et al.* 2011). However, the EPF test is still time-consuming and unreliable.

Cytokines

Cytokines are expressed in a variety of cell types of the reproductive system, although in most instances their functions are not understood. There are, however, a few instances where a role in early pregnancy was established (Schäfer-Somi 2003). First, preimplantation conceptuses of ruminant ungulate species, such as cattle, sheep and goat, secrete a unique type I interferon (IFN-tau). By mechanisms that are still unclear, IFN-tau prevents the destruction of the corpus luteum and hence ensures the continued production of progesterone, which is essential for continuation of pregnancy (Moor *et al.* 1966a, Moor *et al.* 1966b, Martal *et al.* 1979, Capon *et al.*1985, Bazer *et al.*1986, Bazer, 1989, Bazer *et al.*1989, Gnatek *et al.*1989, Bazer *et al.* 1991, Cherny *et al.* 1991, Homeida and Al-Afaleq, 1994). Most likely the IFN-tau prevents luteolysis by modulating the output of a luteolytic hormone, prostaglandin F_2 alpha, from the uterus. In the sheep, Spencer *et al.* (1995a, 1995b) demonstrated, that roINF-τ prevents luteolysis by inhibiting increase in endometrial estrogen and oxytocin receptor gene expression; oxytocin receptor affinity proved to be not affected by roIFN-τ (Spencer *et al.*1995c). Besides anti luteolysis, ovine IFN-τ is thought to exert an antiviral effect via stimulation of 2,5-oligo adenylate synthetase (OAS) expression in the endometrial stroma and glandular epithelium of the early pregnant ovine uterus. The enzyme expression is assumed to be directly regulated by conceptus IFN-τ and to require the presence of progesterone. OAS is additionally thought to participate in the control of cellular growth and differentiation (Johnson *et al.* 2001). oIFN-τ is supposed to be stimulated in part by maternal GM-CSF. Imakawa *et al.* (1993) found that GM-CSF mRNA is localized in the luminal and glandular epithelium of the uterine endometrium and in the conceptus. Despite this involvement in pregnancy, the IFN-τ possess similar antiproliferative and antiviral activities to other Type I IFN, 1 lambda e.g. IFN-alpha. There are 4–5 genes for IFN-tau in sheep whose promotor regions are highly conserved and distinct from those of other Type I IFN. These genes are not virally

inducible and are expressed only in the trophectoderm (outer epithelium of the developing placenta) from the time of blastocyst hatching to implantation. Leukemia inhibitory factor (LIF) is a multi-functional cytokine, which is expressed by uterine endometrium of pregnant mice around day 4 of pregnancy. Female mice lacking a functional LIF gene are fertile but their blastocysts fail to implant, strongly implicating the cytokine in maternal control of implantation. Colony stimulating factors (CSF) are a family of proteins (GM-CSF, CSF-1, G-CSF, and IL-3) that stimulate the cellular proliferation and induction of terminal differentiation of hemopoietic progenitor cells. CSF-1 is expressed in the uterine endometrium of the mouse and human during early pregnancy and its receptor, is present on trophoblast. The osteopetrotic mouse, which represents a natural "knockout" of the CSF-1 gene, exhibits a low rate of fetal implantation and poor fetal viability. It seems likely that CSF-1 expression by the uterus influences growth and differentiation of the placenta. Although different species may utilize different strategies for ensuring developmental and endocrinological coordination between the embryo and the mother, cytokines are likely to be major participants as autocrine factors that direct the events of early pregnancy and not simply as modulators of the maternal immune system (Mathialagan and Roberts 1994). The INF-τ is produced in large amounts by the embryo after day 14 to signal the mother and establish the pregnancy (Roberts 2007). The INF-τ secretion is transitory. It reaches a maximum by 20 to 24 days and is completely gone by day 30 of pregnancy. The IFN-τ is unlike hCG because its expression is transitory and it does not accumulate in the blood or urine. Thus, IFN-τ cannot be used for a pregnancy test in the blood or urine of the small ruminants (Lucy *et al.* 2011). Although the INF-τ cannot be assayed directly in blood, its presence can be detected through its action on leukocytes (white blood cells). Interferon-τ is a cytokine, a class of molecules that has the capacity to stimulate leukocyte function. The leukocyte response to INF-τ can be monitored by measuring the expression of secondary proteins that are called "interferon-stimulated genes" (ISGs) within leukocytes (Gifford *et al.* 2007). Despite their promise as a diagnostic tool for pregnancy detection, the ISGs have not seen commercial application. The current and most reliable assay method involves RNA extraction and RTPCR. The RNA in leukocytes is a highly labile molecule and blood samples would theoretically require special handling before analysis. The RTPCR is a cumbersome and time consuming laboratory process (relative to ELISA, for example). A breakthrough in this area may come if the protein instead of the RNA for ISGs could be measured in blood or if IFN-τ itself could be measured in blood. Blood proteins are typically more stable than blood RNA and may be more suitable for on-farm applications (Lucy *et al.* 2011).

Vaginal biopsy

Histological assessment of the number of layers of the stratified squamous epithelium of the vaginal mucosa obtained by biopsy can be used as a method of diagnosing pregnancy to a limited extent in sheep (Richardson 1972). The basis for the test is the decrease in the layers of the *Stratum germinativum* (vaginal epithelium cells: to 3 to 4 layers at 18–25 days of pregnancy) under the influence of progesterone. The number of layers is high at estrus (around 20 layers) due to influence of estrogen hormone. In sheep the technique was 91% accurate in diagnosing pregnancy after 40 days and the accuracy increased to 100% after 80 days of gestation (Richardson 1972). The limitations of such a diagnosis are the invasive nature of the test, poor results due to improper sampling and improper tissue processing. With the availability of more precise techniques of pregnancy diagnosis in sheep and goats, the use of vaginal biopsy for pregnancy diagnosis has reduced (Purohit 2010).

Conclusions

The development of a reliable and accurate pregnancy diagnosis test for use on farm animals for the early diagnosis of pregnancy would enable the prompt rebreeding of non-pregnant animals and prevent the culling of pregnant animals in error. Accurate pregnancy diagnosis can be achieved by progesterone and PAG assays, however, their accuracy for differentiating single and multiple fetuses would not be regarded as sufficiently high to be of practical value and they are expensive. Rectal abdominal palpation is a simple, cheap and quick method, however its accuracy for determining multiple pregnancies is low and it may cause abortion or rectal perforation. Doppler technique requires great skill to achieve high accuracy for prediction of fetal numbers. Radiography and transabdominal B-mode ultrasonography accurately diagnose both pregnancy and fetal numbers, but the second technique is cheaper than the first one and has the advantages of being safe and able to detect the fetal viability.

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