Methods Of Pregnancy Diagnosis In Domestic Animals: The Current Status

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Abstract

Methods of pregnancy diagnosis in the domestic animals can be classified into visual, clinical and laboratory methods. In most domestic animals clinical methods are currently used. Visual methods are far from perfect in domestic animals. In animals like cattle, buffaloes and mare’s recto genital palpation and trans-rectal ultrasonography continue to be the methods of choice for an accurate and early pregnancy diagnosis. In sheep, goat, sow, bitch and cat ultrasonography is the only reliable method of pregnancy diagnosis. In the camel cocking of the tail is an effective visual method of pregnancy diagnosis and recto genital and ultrasonography are also useful. Laboratory methods used in past and developed in recent years are described. The efficiency of a few of these procedures is likely to be improved in near future.

Introduction

The diagnosis of pregnancy (cyesiognosis) has been sought since long by farmers for curiosity however, it is essential for profitable animal husbandry especially in the productive animal species. For an economical dairy farm, cows must calve every year, and to maintain this sequence, identifying pregnant animals at an early date seems imperative. In the current systems of planned breeding, diagnosis of pregnancy would help to evaluate the therapies at an early date and devise alternative manipulations. In some situations in the pet practice pregnancy may not be desirable by the owners and an early diagnosis would help in termination of these unwanted pregnancies. An early pregnancy diagnosis is essential in mares to tease them if non pregnant, and try to get them pregnant in the same season. It therefore, appears that early diagnosis of pregnancy is essential in animal management for economic reasons. In many developing countries, farmers often present their animals for pregnancy diagnosis very late when much of their time is lost in maintaining non pregnant cows.

There is a need to educate farmers to get their animals checked for pregnancy at an early date as it has been shown that earlier the pregnancy diagnosis performed, the more profitable is the return for dairy cows and buffaloes (Oltenacu, 1990; Duggal et al., 2001a; Youngquist, 1997).

Review

Methods of Pregnancy Diagnosis
A variety of approaches have been evaluated and developed during the past and recent years, some of which have some limitations to their wide scale use. The methods of pregnancy diagnosis have been classified into two (direct and indirect) or three categories i) Visual methods, ii) Clinical methods, iii) Laboratory tests.

Visual Methods
Non return to estrus
When an animal is mated and it does not return to estrus the owner usually thinks that the animal has become pregnant and hence has not returned to estrus. This happens because during pregnancy, the conceptus inhibits the regression of the corpus luteum and thus, prevents the animal from returning to estrus. However, many a times the animal does not return to estrus because of non regression of CL due to reasons other than pregnancy. Moreover, in the seasonally breeding species like sheep, goat and mares the ability to detect estrus may not return to estrus (when mating is done during the end of the breeding season) because the season was over. Anoestrus, and the rare occurrence of gestational estrus in cattle and buffaloes can affect the reliability of non return to estrus as a method of pregnancy diagnosis. Moreover, difficulty in estrus detection and silent estrus render this method of pregnancy diagnosis unsuitable for the buffalo. Likewise, estrus detection methods used for sheep, goat and mares need to be properly designed so as to make efficient use of non-return to estrus as a method of pregnancy diagnosis in these species. In dairy cows non-return rates usually over estimate true pregnancy diagnosis (Kidder et al., 1954) and are also affected by the detection procedure used (Foote, 1974). Moreover,
estrus expression appears to be reduced in intensity and duration in the present day dairy cows leading to lower estrus detection efficiency (Dransfield et al., 1998; Lucy, 2001). Therefore, the probability of misdiagnosis of pregnant females by estrus observation appears to be increased. This may be confounded by a small proportion of pregnant cows and buffaloes expressing estrus (Gilmore, 1952; Agarwal and Tomer, 1998) and some cows expressing prolonged inter-estrus intervals of around 24 days (Sartori et al., 2004). Therefore, non return to estrus is an unreliable procedure for pregnancy diagnosis in most domestic animal species. Besides the non-return to estrus a few of other visual signs of pregnancy appearing in late pregnancy include increase in the size of the abdomen, development of the udder specially in dairy heifers (4 months onwards), slight vaginal discharge (from 4-5 months onward in dairy cows) and movements of the fetus visible externally (specially in fed cows on the right side of abdomen 6 months onwards). However, the accuracy of these visual diagnostic symptoms is always low and a clinician must use them as a supplement to clinical diagnosis.

**Cocking of the tail**

The pregnant female dromedary camels exhibit a characteristic behavior when approached by a male or a person. The female assumes a stiffened posture with the head held high and tail curled upwards. (Banerjee, 1974; Banerjee et al., 1981). This is known as cocking of the tail. This behavior appears 14 to 15 days after fertile mating and known to be 95% reliable for pregnancy diagnosis in quiet and calm dromedary females camels. However, many false positives can be obtained in agitated females if the observer is untrained. Tail cocking is also observed in the pregnant Bactrian camel although not with the same intensity as in the dromedary female camel (Tibary and Anouassi, 1997).

**Clinical methods of pregnancy diagnosis**

Four clinical methods of pregnancy diagnosis are available for pregnancy diagnosis in the various domestic farm and pet animal species i) rectal palpation, ii) abdominal ballottement, iii) ultrasonography and iv) radiography. Each of these methods is discussed separately.

**Recto-genital palpation**

Transrectal palpation is the oldest and most widely used method for early pregnancy diagnosis in dairy cattle (Cowie, 1948). In most large domestic animal species like cattle, buffaloes, mares and female camels recto-genital palpation (with some limitations) is the easiest, cheapest and fastest method of pregnancy diagnosis with little or nil harm to the animal and its fetus when performed carefully. To a limited extent this method is used for pregnancy diagnosis in pigs. The method is the technique of choice being taught to veterinary graduates and para veterinary staff. Transrectal palpation is considered to be an accurate method of pregnancy diagnosis in dairy cattle for a trained veterinarian after day 35 post breeding (Euler, 1930; Gotze, 1940; Roberts, 1971; Zemjanis, 1970; Momont, 1990). A few studies point out that the procedure may increase the risk of iatrogenic embryonic mortality in dairy cattle (Paisley et al., 1978; Vaillancourt et al., 1979; White et al., 1989) while others and the author do not concur with this view. The procedure however, does not provide any information about the viability of the embryo/fetus during earlier stages of pregnancy (Romano and Magee, 2001). Some of the basic principles which a palpator must understand and the precautions that he must observe are mentioned below and then the findings are described for cattle, buffaloes, mare, camels and pigs. Transrectal palpation of the amniotic vesicle as an aid in determining pregnancy status in dairy cattle was described by Wisnicky and Cassida (1948), whereas slipping of the chorioallantoic membranes between the palpator’s thumb and forefinger beginning on about day 30 of gestation was described by Zemjanis (1970).

**Basic principle**

The genital organs lie usually on the pelvic floor during early pregnancy beneath the rectum in most species and in the abdominal cavity during late gestation. The genital organs can thus be palpated indirectly by placing the hand in the rectum evacuated of the feces. The growth of the conceptus in either of the uterine horn leads to sequential increase in the size, tenseness and palpable characteristics of the uterine cornua. Thus, with experience the palpator can feel these changes in the uterus of a pregnant animal and with fair to good accuracy predict pregnancy depending upon the species, stage of gestation and his experience. Two bottle necks appear to be significant while performing rectal palpation, i) the peristalsis that occurs in the rectal musculature, which produces obstacles in palpation and ii) ballooning of the rectal wall due to entry of air inside. The palpator must stop making movements of arm during a peristaltic wave (while still keeping his hand inside the rectum) wait for 1-2 minutes and then start palpation again when the peristalsis has subsided. The ballooning of rectum can be easily appreciated, by the finding that the operator can move his hand up and down in the rectum without resistance when the rectum is ballooned. The operator must catch hold of a pinch of rectal mucosa and move his hand back and
forth (known as back racking) without completely taking it out. This will push the air inside, to the exterior and the rectal mucosa will then be closely over the operator's hand.

After proper restraint and wearing of proper clothing and also proper lubrication, the operator must make a cone of his hand and push it inside the rectum. The anal sphincter dilates and the hand enters inside the rectum. The feces must be removed without taking out the hand completely. The cervix which is a hard round to oval or sometimes caudally enlarged disfigured structure is the land mark for location of genital structures in cattle and buffaloes. This can be located by sliding the hand in an arc like fashion from dorsal to ventral side. The cervix is followed further to locate the uterine body and the uterine horns. These structures can be pulled caudally when located at the pelvic brim or further, by retracting the broad ligament or hooking the inter-cornual ligament by the index finger. When the pregnancy is beyond 60 days this cannot usually be done and the operator has to move his hand further in the rectum, so as to locate the intra-abdominally placed uterus and palpate other features diagnostic of pregnancy.

In the mare, the cervix is not easily palpable and hence the ovaries are the land mark for rectal palpation especially for novice palpators. They are located about 10-20 cm cranial to the shaft of the ilium bone and about 5 to 10 cm below the lumbar vertebrae in non-pregnant mares, and in mare during early pregnancy. After locating one ovary the hand is passed down the utero-ovarian ligament to locate the uterus.

A striking feature of the genitalia of the female camel is the shortness of the right uterine horn. This is probably, because of the existence of exclusive left horn pregnancies in this species (Tibary and Anouassi, 1997). Recto-genital palpation is similar to that described for cattle and buffalo as the cervix is easily palpable.

Rectal palpation is only possible to a limited extent in the large sized sows. It is barely possible in gilts and in small sized breeds of sows.

Restraint and clothing

The animal to be examined should be properly restrained. Cows and buffaloes can be securely restrained in a Travis or chute. At many situations when this is not available the hind legs of cows are tied with a rope to avoid kicking and the head is held securely. The tail is held to one side by an assistant. Pressing on the back relaxes the pelvic structures and reduces peristalsis. Buffaloes often resist tying ropes on hind legs and ropes are usually tied either on both fore legs or one leg while folding it from the knee. Buffaloes also kick less frequently by hind legs but, sometimes excitable, nervous buffaloes do not allow rectal palpation by moving frequently. Such animals may rarely need little sedation, examination in a sitting position or examination at a later date. Palpators must take every care to avoid damage to themselves by the kick of the animal.

Thoroughbred mares are usually docile and can be easily made to enter a chute however; some nervous mares or mares of other breeds need different restraint procedures. It is often safe for the palpator to make the mare stand on one side of a small wall (3 ft) and the examiner stand on the other side of the wall. Stable wooden rails can sometimes serve the purpose. A good approach would be to tie both hind legs with one fore leg using rope. A twitch on the lower lip, ear or nose and/or lifting one fore leg may be required. Nervous mares sometimes move sideways or try to jump resisting any examination. Such mares need tranquilization.

Female camels are examined in a sitting position with both hind, and forelegs tied together separately with ropes. An assistant holds the head tightly. Vicious females often require pressure on the back by legs of persons standing on both sides to prevent side wise movements.

The examiners must wear proper clothing including coveralls, gum boots and disposable plastic or rubber full arm sleeves. This is essential to protect the examiner from contracting zoonotic disease and spoiling his clothes. Separate trousers and shirts made of dark coloured (green or blue) slightly thick cloth are easier for working compared to a single cover all. Plastic long sized aprons are used by many clinicians in the field.

Sufficient lubrication must be used while introducing the hand in the rectum. Non-irritating soap and water or liquid paraffin is a suitable lubricant. Since, the feces of the mare are harder and the peristalsis stronger, more frequent lubrication is essential in the mare. Tail bandaging is also advantageous in the mare to avoid tail hairs enter the rectum and cause damage to the rectal wall.

Precautions during rectal palpation

When performed gently and carefully rectal palpation is a non-invasive procedure. The following points would be helpful in minimizing damage to the animal and the examiner as well.

1) Ruthless movements of the hand in the rectum should be avoided. Avoid palpations during a peristaltic wave.
2) Examiners must trim their nails and avoid using dirty soiled sleeves.
3) Rectal examination without a sleeve must be
avoided specially in mares to avoid contracting diseases and obnoxious odors. Sleeves must be replaced after examination of 2-5 animals, or better after each examination.

4) Rectal palpation of an animal suffering from fever should be extremely gentle or better avoided as the blood vessels are more fragile and bleed easily. Similarly examining an animal with rectal tear or rectal fistula is hazardous. Whenever, a clinician notices such conditions he must bring them to the notice of the owner or else he would blame the clinician. Rectal fistulas are an emergency in a mare and immediate treatment including broad spectrum oral and parental antibiotics and anti-inflammatory drugs must be given along with laxatives or else the mare may develop a fatal peritonitis. The fistula/tear may be palpable as a blind pouch or a slit in the rectal mucosa that bleeds when the hand is forwarded through these openings.

5) Compared to cattle rectal palpation in buffaloes must be gentle as the rectal mucosa is more fragile and bleed easily.

6) Clinicians must assure that even the animal kicks it does not harm them, and so also the palpators must also be cautious that sudden sideways movement of the animal with the operators hand inside can cause fracture of the operators arm and hence due care must be exercised.

7) Uncareful palpation of the uterine horns with undue pressure can cause rupture of the amniotic vesicle and loss of an early pregnancy and hence this must be avoided.

Consequences of improper palpation

Because pregnancy in cattle can be terminated by manual rupture of the amniotic vesicle (Ball and Carroll, 1963), many studies have investigated the extent of iatrogenic embryonic mortality induced by transrectal palpation. Several studies have suggested that examining pregnant cows early in gestation by transrectal palpation increases the risk of iatrogenic embryonic mortality (Abbitt et al., 1978; Franco et al., 1987; Paisley et al., 1978; Valliancourt et al., 1979; White et al., 1989), whereas other studies have suggested that cows submitted for transrectal palpation earlier during gestation had a decreased risk for abortion or that palpation had no effect on subsequent embryonic losses (Studer, 1969; Thurmond and Picanso, 1993). Although controversy still exists regarding the extent of iatrogenic embryonic mortality induced by transrectal palpation, other factors have a greater influence on calving rates than pregnancy examination by transrectal palpation (Thompson et al., 1994). Furthermore, because the risk of embryonic mortality is high during the period of gestation when cows are diagnosed pregnant by transrectal palpation, and because most cows within a herd are submitted for pregnancy examination, it is impossible for dairy producers and veterinarians to distinguish between iatrogenic losses occurring due to transrectal palpation and spontaneous losses that would normally have occurred in these cows.

Palpable findings of pregnancy in cattle and buffaloes

Palpable findings of pregnancy have been described in detail previously (Roberts, 1985). The water buffalo has an approximately one month longer gestation period compared to cattle however; the parameters used in cattle are used in the water buffalo for pregnancy diagnosis by rectal palpation.

Two things must be kept in mind by clinicians in making positive diagnosis of pregnancy by rectal palpations in cattle and buffalo. The first is, that when the palpator in unable to detect any of the palpable characteristics mentioned herein, he must neither comment positively or negatively as both would be frustrating both to the clinician and the owner on a later date. He must better admit the fact that he is not able to detect out properly and the animal must be re-submitted for examination 15-30 days later preferably after a fasting. The second thing that clinicians must keep in mind is the accuracy of the gestation period (this is especially applicable for the 5-8 month period in cattle and 5-9 months in buffalo).

An approximation of the gestation period must be conveyed to the owner rather than an accurate period. Although experienced clinicians can more precisely comment on the gestation period after an examination it is usually safe to be approximate.

When pregnancy examinations are made early (Day 30-45) the possibility of an early embryonic death must be kept in mind and a confirmation of pregnancy must be done only after re-examination at a later period (60-90 days).

Owners often consult veterinarians on the fetal viability during mid to late gestation. It is often difficult to comment positively by a single rectal palpation. Until unless characteristic changes in the uterus and fetus are palpable negative comments must better be avoided. The usual test for fetal viability during this period is the movement of the fetus in response to a stimuli by the examiners hand (movement of a fetal leg when pressed by hand or suckling movements by the calf when a finger is touched in the mouth) however, this may be sometimes misleading specially when the fetus is depressed.

Location of pregnant uterus

The pregnant uterine horn under goes sequential changes in size, location and morphology and they form the basis for pregnancy diagnosis. The
The entire diameter of each uterine horn must be connective tissue band. The pressure must be gentle before the uterine wall escapes. It is felt like a allantois chorion, slip between the thumb and fingers to slip while the fingers compress it gently. The uterine horn must be grasped in the palm and allowed 35-90 days of gestation (Zemjanis, 1970). The entire fetal membrane slip can be felt between fingers (9.0 cm) at 60-62 days of gestation (Roberts, 1985).

### Slipping of the fetal membranes

The fetal membrane slip can be felt between 35-90 days of gestation (Zemjanis, 1970). The entire uterine horn must be grasped in the palm and allowed to slip while the fingers compress it gently. The allantois chorion, slip between the thumb and fingers before the uterine wall escapes. It is felt like a connective tissue band. The pressure must be gentle. The entire diameter of each uterine horn must be palpated.

### Palpation of placentomes

The presence of placentomes is another positive sign of pregnancy and is detectable from about 75 days to term. The period of pregnancy when the uterus has descended into the abdominal cavity and the fetus is not palpable, palpation of a placentome is the surest indication that the cow/buffalo is pregnant. Since there is great variation in size among individual placentomes (those nearest the fetus are the largest), their usefulness in aging a pregnancy is limited. In general, they can be detected as soft, thickened lumps in the uterine wall and are more easily detected as pregnancy advances.

### Palpation of the fetus

The palpation of the fetus itself is a positive sign of pregnancy. Depending on the skill of the examiner and the location of the fetus, the uterus horn is palpable from about 100 days of pregnancy. However, in large cows the abdomen should be lifted by a hand directed laterally towards the iliac shafts reflecting in a cranio ventral direction. They can thus, be felt by the hand directed laterally towards the iliac shaft. These vessels are palpable within the pelvic cavity or just cranial to the pelvic brim. Palpation of a fetal extremity is sufficient evidence for pregnancy if other uterine findings are normal.

### Palpation of uterine artery fremitus

The major supply of blood for the gravid uterus arrives via the uterine arteries, which enlarge considerably as pregnancy progresses. These bilateral vessels travel in the broad ligaments, just below and anterior to the iliac shafts reflecting in a cranio ventral direction. They can thus, be felt by the hand directed laterally towards the iliac shaft. These vessels are freely movable. Enlargement of the uterine artery ipsilateral to the pregnant horn is detectable after 80 to 90 days of gestation. By approximately 120 days, the blood flow within the artery increases to a point where the blood flow is palpable as a buzzing sensation, also called "thrill" or "fremitus". By 7 to 8 months the fremitus is often palpable on the side of the non-pregnant uterine horn also. The detection of...
fremitus is a positive sign of pregnancy.

**Palpation of ovaries**

As the pregnancy advances ovaries may be dragged forward along with the pregnant uterus and may not be palpable beyond the four to five months. Vaginal changes

During pregnancy the vagina usually develops a pale, dry sticky mucus membrane. The cervix is closed and the cervical (mucus) seal covers the external os by day 40 to 120 of pregnancy. Slight degree of vaginal discharge is evident in some cows beyond 5 months of pregnancy but the cervical seal liquefies only prior to parturition or abortion and is discharged in strings.

**Differential Diagnosis of pregnancy**

Owners are many times perplexed on the outcome of a pregnancy that did not progress normally and often present their animals for a definitive diagnosis to a clinician. Some pathological conditions mentioned in detail in subsequent chapters often should be identified to be different from a normal pregnancy. The palpable differences of these conditions are briefly described below.

Only rarely does a urinary bladder full of urine creates confusion for the presence of an early pregnancy (2-3 months) in dairy cows. This can be easily differentiated by the absence of palpation of both uterine horns and the ease with which the animal urinates when the bladder is gently massaged leading to disappearance of the enlargement. Rarely tumors of the genital tract can create confusion, but their consistency and location is different. The differential diagnosis of three conditions commonly encountered by clinician’s namely mummified fetus, macerated fetus and/or pyometra/mucometra is described below:

**Mummified fetus**

A uterus with mummified fetus has thick uterine walls, absence of fluid and placentomes and a hard palpable structure. The fetus is closely apposing the uterine wall. The fremitus is absent.

**Maceration of the fetus**

There is copious vaginal pus discharge. The uterine wall is thick and doughy. There is no dorsal bulging of the uterus and placentomes are not palpable. Parts of bones are sometimes palpable separately floating tucked up. The fremitus is absent.

**Pyometra and Mucometra**

These two conditions are many times difficult to differentiate from normal pregnancy especially when the pus or mucus is present in enormous quantity (sometimes 20-40 liters) so that the uterus is largely enlarged and placed on the abdominal floor. In pyometra the uterine wall is thick, uterus is doughy and placentomes or fetus is not palpable and the fremitus is absent. However, it is always safer to make a re-examination 1 to 2 months later when the clinician is not sure, or make use of ultrasonography to differentiate the condition. Sonographic evaluation would depict anechoic fluid without cotyledons and echogenic material accumulated. A clinical therapy to terminate the corpus luteum on an assumption that the condition is pyometra could be hazardous and loss to the owner, if unfortunately the condition was a normal pregnancy. In mucometra, the positive findings of pregnancy are absent, but, contrary to pyometra the uterine wall is thin. Ultrasonography can easily differentiate the condition from pregnancy. When these two conditions are to be differentiated from early pregnancy a characteristic feature found most often is the bilateral enlargement of both uterine horns, which is not found in pregnancy. The fetus is not palpable in these two pathologies, and often there is a history of vaginal discharge.

**Palpable findings of pregnancy in mares**

The early diagnosis of pregnancy in the mare has its own significance. A limited breeding season in some breed registries (for e.g. Thoroughbred) warrants that the non pregnant mares are diagnosed early and steps be taken to breed the mare again in the same breeding season. Some peculiarities of the equine pregnancy are that an ovarian source of progesterone is essential for maintenance of pregnancy until approximately days 50 to 70; thereafter, the fetal-placental unit begins producing sufficient progestagens to support pregnancy. (Bergfelt and Adams, 2007).

The endometrial cups form at about 35 days of gestation and remain up to 150 days. Secondary, accessory and supplemental corpora lutea (day 40) form on the mare ovary and all corpora lutea regress by day 180-210 (Bergfelt and Adams, 2007). The early equine fetomaternal axis secretes sufficient estrogen and the equine embryo stabilizes by day 16.

Diagnosis of an early pregnancy by rectal palpation is one of the easiest and accurate means for experienced personnel. However, a diagnosis at day 18-20 should be confirmed at day 45-60 because of a late implantation of the equine embryo and chances of early embryonic death and reabsorption. The basis of pregnancy diagnosis in the equine species lies on the changes that follow in the cervix, uterus and ovaries. Placentomes are not found in the equine species and the fremitus or fetal membrane slip is not marked, therefore some of the parameters used in cattle cannot be used in the equine species directly.

**Cervix**

As early as 16 to 18 days after ovulation, the cervix of the pregnant mare becomes tightly closed,
firm, slender, and elongated. Between 16 to 30 days the cervix can be with experience palpated on the floor of pelvis as a rigid firm structure.

Uterus

Most pregnancy diagnosis in the mare by rectal palpation is done by palpation of the uterus. The parameters used for a positive diagnosis are as follows-

Uterine tone and thickness

One of the positive sign that a mare has conceived to a breeding is the finding of increase in uterine wall thickness and a marked tone. The endometrial folds are no longer palpable as folds of tissue. The uterus becomes tubular, smooth and firm. The uterine tone appears at days 15-16 and continues up to day 48 to 55 (Sertich, 1997). This is diagnostic except in cases of endometritis or in mares bred at the foal heat, which are still undergoing uterine involution.

Uterine size and embryonic vesicle

The conceptus becomes positioned at the base of one of the uterine horns at the junction between uterine horn and body. At 25-28 days, a small bulge may be palpable in maiden mares. The bulge progressively increases in size and is palpable in most mares by day 30-35 (approx 3-4 cm in diameter). A small notch can be readily appreciated on both sides of the bulge. By day 42-45, the conceptus occupies approximately half of the gravid horn and is 5 to 7 cm in diameter. The uterine wall over the bulge is thin.

At 60 days of gestation, nearly the entire gravid horn and half of the uterine body are filled with the conceptus but the non-gravid horn remains small. The pregnancy is like an elongated football and is nearly similar to a 60 day pregnancy in cattle. The tonicity is markedly reduced at this time. The 60 day conceptus is approximately 8-10 cm in diameter and 12 to 15 cm in length.

Palpation of fetus

The fetus is active after 40 days and mobile after 70 days. Palpation of the turgidity of the conceptus is absent by day 90, and the fetus is palpable, which feels like a small, heavy, submerged but floating object as the hand contacts it. In most mares it is usually possible to palpate the fetus per rectum from the third month onward throughout the gestation. In a few deep bodied and large sized mares palpation of the fetus is difficult from the fifth to seventh months of gestation. In these mares the location of uterus and ovaries would aid the diagnosis.

Location of the uterus

The uterus is located in the pelvic cavity or just at the pelvic brim until day 90. At 100-200 days, the gravid uterus is positioned cranial to the pelvic brim in the abdominal cavity (Sertich, 1997). The ovaries are positioned cranial and ventral to their normal positions and closer together.

By 5 to 7 months the uterus is positioned low in the abdomen and it is difficult to thoroughly evaluate the conceptus by palpation per rectum. During the sixth and seventh months, the horns are approximately perpendicular to the dorsal cranial aspect of the uterine body. Beyond the seven to eight months the fetus is easily palpable by rectal palpation.

Ovarian palpation

Both ovaries should be palpated. Both ovaries usually are enlarged from 18 to 40 days as a result of follicular development and the CL is not palpable. From 40 to 120 days, extensive ovarian activity with ovulations, luteinization, and development of secondary corpora lutea is evident. Follicular activity decreases from 120 days to term, and the ovaries become small and inactive. The position of the ovaries up to 60 days of pregnancy is similar to that for the non-pregnant mare. From then on, they are drawn cranially and medially but remain dorsal to the uterus. The finding of both ovaries nearer to each other and close to the pelvic floor is a positive indication for pregnancy when the uterus or other structures are difficult to palpate (3 to 5 months). Form 5 months of pregnancy onwards, the ovaries usually are not palpable as they are under the broad ligaments.

Rectal palpation in the sow

To a limited extent rectal palpation has been described as a method of pregnancy diagnosis in the sow. (Cameron, 1977). Sows are examined while standing in gestation crates or pens. This technique is based on examination of the cervix and uterus, together with palpation of the middle uterine artery to assess size, degree of tone, and type of pulse. At around 21-30 days of gestation the bifurcation of the cornua is less distinct, the cervix and uterine walls are flaccid and thin. The middle uterine artery is 5-8 mm in diameter and more easily identified. The uterus becomes progressively thin walled and ill defined by 31-60 days and fremitus can be identified at 37 days. Beyond 60 days the fremitus is very strong, however, piglets can only be palpated towards the end of gestation. The procedure is often difficult in gilts and small sized breeds because of a small pelvis and too small rectum. False positive diagnosis is likely if the external iliac artery is mistakenly identified as the middle uterine artery. The technique is however, not popular at most locations.

Rectal palpation in female camels

The corpus luteum formed on the camel ovary (ovulation is induced by mating) persists and is necessary for the entire gestation. The persistence of the CL is one of the earliest sign of pregnancy as
otherwise; the luteal phase is very short. The CL is out of reach by day 90 of pregnancy. The left uterine horn is inherently longer than the right horn and this must be kept in mind when making pregnancy diagnosis in female camels. The earliest detection of uterine change (increase in diameter and appearance of fluctuation) is palpable at about 40-15 days. Between 60-70 days, the left uterine horn is increased about twice to its non gravid size, has a thin wall and fluctuates (Banerjee, 1974).

The gestational changes in camel have been described (Tibary and Anouassi, 1997). The uterus becomes cranial and ventral after the third month of pregnancy. The cervix is pulled forward and lies just at the pelvic brim by 4 months. At 5 months of pregnancy, the uterus is completely in an abdominal position with a small degree of fluctuation but the fetus is not always palpable. The fetus becomes palpable again beyond the 6th month of pregnancy, first with ballottement, and then, the head and legs become easily palpable by the 7-8 months as the fetus starts its ascent. By the 9th month, movement can be observed by inspection of the right flank of the animal. At around 11 months the fetal legs can be easily found in the pelvic cavity. The precise estimation of the stage of pregnancy beyond 3 months, because of the absence of cotyledons and difficulty in reaching the fetus in this species is difficult. The fetal membrane slip is not seen in camels because of a diffuse placenta.

Ultrasoundography

During the last couple of years, ultrasonography has gained popularity in veterinary medicine and has become the method of choice for diagnostic imaging of the various organs of the body, including reproductive organs. Ultrasound is a high frequency sound wave. Sounds audible to the human ear vary between 20 to 20,000 Hertz (Hz) (Cycles per second) while ultrasound waves are of frequency higher than this, and for most diagnostic applications frequencies of 1-10 MHz are used. Ultrasound cannot be propagated in vacuum and in gas, transmission is poor. Reflection of ultrasound occurs between substances of different acoustic impedance (defined as the product of the velocity of sound in a substance and the density of the substance). Even the short distance between the transducer (which emits and receives ultrasound signals) and the patient must be bridged by a suitable coupling gel.

Basic principle

The ultrasound equipment basically consists of a transducer and a scan converter. The transducer is the ultrasound producing part. It is fitted with a piezoelectric crystal (Lead – zirconate – titanate or others) which when stimulated by a high voltage current emits the ultrasound. The ultrasound is transmitted to the patient from the transducer and propagates through the tissues. The ultrasound beam is either reflected back, partially absorbed or entirely absorbed. The returning beam (echoes) meets back and deforms the crystals in the transducer. This mechanical energy is converted back to an electrical signal proportional to the strength of the echo and delayed by a time roughly proportional to the distance traveled. The scan converter interprets the variations in brightness displayed on the cathode ray tube of a B-mode system (or as a variation in amplitude in A-mode oscilloscope screen) and also stores images when required. The ultrasound is emitted in a pulse – echo manner. A pulse of ultrasound is emitted and its reflection perceived prior to emission of the next pulse.

Types of instruments and some definitions

For most diagnostic veterinary purposes B-mode, real time ultrasonography is used employing different types of transducers. Transducers used commonly in veterinary reproductive practice are the linear transrectal transducer (frequencies of 5-10 MHz) and the sector transabdominal transducer (frequencies of 1-4.0 MHz). For most reproductive diagnostic work, linear array transrectal transducers are employed in cattle, buffaloes, mares and female camels. Small sized transrectal transducers are also used for early pregnancy diagnosis in small ruminants (sheep and goat).

For bitches mostly transabdominal sector transducers are useful for pregnancy diagnosis with frequencies from 2 to 4.0 MHz. The same transducers can be used for pregnancy diagnosis in sheep and goat beyond day 40 of gestation. However, in order to visualize an early pregnancy or the non pregnant bitch uterus transducers of high frequency (5-7.5 or 10.0 MHz) are essential.

A wide variety of transducers with single, dual, or multiple frequency and multiple functions are available and clinicians must decide what type of instrument and the transducer he must purchase depending upon his work. Advancement to ultrasonography includes diagnostic imaging using color doppler, magnetic resonance imaging and readers must refer pertinent excellent texts available elsewhere (Singh et al., 2003).

When performing ultrasonography it is important for the sonographer to have basic knowledge of the anatomic location of the different organs to be visualized and problems that can be encountered in obtaining and interpreting the images obtained. A few of the common terms related to ultrasonography are described below:-
Anechoic (sonolucent)  A tissue failing to reflect the ultrasound beam produces no echoes (e.g. A fluid filled follicle) and appears black.

A- mode  Amplitude modulation. A one-element (one dimensional) display with time (distance) on the horizontal axis.

B-Mode  Brightness modulation. A compound A-mode scan with amplitude translated into a brightness scale. Location on the display is related to position and depth.

Doppler ultrasound  When an ultrasound beam meets a moving object the reflected ultrasound is either of increased or decreased frequency, depending upon whether the motion is towards or away from the transducer.

Echogenic  A structure causing a marked reflection of the ultrasound beam. A change in echogenicity in a homogeneous structure may indicate a pathological change.

Gain  The amplification level of a returned signal.

Hyperechoic  Showing increased echogenicity.

Hypoechoic  Showing decreased echogenicity.

Linear array  Distribution of piezoelectric crystals along the length of a scan head. The image produced is generally rectangular.

M-Mode  Motion mode. A rapidly updated one dimensional B-mode display with time on the second axis to allow study of moving structures. Used in cardiology.

Probe  The transducer and its attachments.

Real time  Images generated from reflected ultrasound following sequential activation of transducer array are displayed on the screen at sufficient speed to give the appearance of a live image.

Scatter  When the ultrasound beam encounters a small object in its path the beam energy is spread in all directions.

Sector scan  A pieslice/sector shaped image is produced on the screen. The initial signal is produced by a single or small number of rotating piezoelectric crystals.

Shadowing  Caused by severe attenuation (decrease in the power of ultrasound beam by absorption, scatter and reflection) such that it fails to penetrate sufficiently deeply.

Transducer  The piezoelectric crystal or element which converts electrical to mechanical energy. Procedure for ultrasonography

Transrectal ultrasonography  The detailed methods for performing transrectal ultrasonography in cattle have been described in detail previously (Goddard, 1995 Manion, 2006). The animal to be examined is properly restrained, the feces are evacuated from the rectum and the perineum washed with water. The transducer is covered with a condom with coupling gel put inside or a full arm disposable sleeve is used to cover the transducer. The operator keeps the transducer in his arm and takes it inside the rectum. The uterine horn on one side is scanned to the entire length and the ovary of that side is also scanned. The operator then moves his hand to the other uterine horn and ovaries. If the pregnancy is advanced the operator may have to take his hand deeper. When required the images seen may be frozen and the diameter of the structures measured by inbuilt calipers with the machine. The amount of fluid and thickness can also be measured. The transducer is then taken out and the perineum washed again.

Trans-abdominal (Transcutaneous) ultrasonography  For reproductive trans-abdominal ultrasonography in sheep and goats the hair must be clipped from just above the udder and 15 to 20 cm ahead of the udder on both sides of the abdomen. The transducer is placed above the udder between the thigh and abdomen preferably the left side and moved in a ‘W’ shape from one side of the abdomen to the other side. The procedure can be performed with the animal standing (sector scan) or in lateral or ventral recumbency (linear scan) depending upon the type of transducer being used.

For pregnancy diagnosis, sows are examined in a standing position when using sector scanners (3.5-5.0 MH2 frequency) or doppler instruments (which are more frequently used in pigs to detect fetal heart beat, fetal movements and uterine artery pulsations). The transducers are placed over the abdomen just medial to the stifle skin fold, just at the level of second last teat.

Trans-abdominal imaging of the uterus of a bitch can be done with the bitch in the standing position after clipping the hair of the ventral abdomen. However, dorsal and lateral recumbent positions may also be used. The uterus lies dorsal to the bladder, but its position may vary with the extent of bladder filling, and the size of the uterus. During early pregnancy the uterus has a more dorsal position in the abdomen of a standing bitch, but with advanced pregnancy the uterus is closer to the ventral lower abdominal wall.

Sonographic findings during pregnancy  Interpretation of sonograms of the reproductive tract requires an understanding of the composition of the images and an awareness of the possible artifacts which can occur and lead to misdiagnosis. For example, acoustic enhancement will appear as a hyper echoic region deep to the fluid (anechoic) area. Gas and bone will totally reflect the sound waves and produce the strongest of echo signals, leading to an
image which appears on the screen as near-white. So complete is the return of echoes in some cases that sound waves do not penetrate deep to these areas, resulting in a lack of imaging which manifests itself as a black zone and is referred to as acoustic shadowing. This must not be interpreted as the anechoic image of fluid filled structures. Another artifact found with gas filled viscera or bone is the rebounding of echoes back and forth between the object and the transducer. With each cycle of rebound there is loss of signal strength, and this is imaged on the screen as a series of layered hyper echoic images repeating themselves between the object and the transducer face. This is termed reverberation and is often encountered in trans-rectal scanning where gas filled viscera are present. When the ultrasound strikes to a smooth and wide structure (for e.g. a CL) there will be almost total return of echoes where the sound waves strike at right angles, giving an intensified signal that appears on the screen as a whiter shade of grey. This is referred to as specular reflection, and is often seen in early pregnancy when imaging the embryonic vesicle.

With these basic things in mind one has also to keep in mind that fluid filled structures appear black (anechoic), hard structures (like the bone) appear white (hyperechoic) and other structures with their structure midway between the bone and fluid appear grey (hypoechoic). The basic diagnosis of pregnancy lies in the identification of structures from black, grey or white scale.

The earliest sonographic finding for pregnancy in most animals is the appearance of anechoic fluid within the uterine lumen. This fluid goes on increasing to a stage when the embryo proper becomes visible as a hypoechoic structure floating within this fluid and progressively the fetal structures become more clearly visible along with the fetal membranes. The viability of a growing fetus is ascertained when the fetal heart beat becomes visible as a hypoechoic flickering structure.

The methods of evaluation of pregnancies using ultrasonography have been described for various species in sufficient detail by Goddard (1995). The days of sonographic appearance of various structures in different domestic animals are mentioned in Table 1. On analyzing this table it is clear that with different probes the amniotic vesicle/fetal fluid is visible in most species between days 18-22 except in the mare in which it appears earlier (day 10-16). The fetal heart beat can be seen between day 24-30 and the fetus itself between days 25-30 in most species. The cotyledons are visible between day 30-40 and fetal extremity/bone by day 57-60 in cattle, day 70 in sheep; however it is visible earlier (42-50 days) in the bitch. It therefore, appears that the fetal fluid, fetal heart beat and the fetus become sonographically visible at nearly the same time in most species. The most appropriate time for pregnancy diagnosis using ultrasonography with high accuracy in cattle, camel and buffaloes appears to be day 28-30 using a trans rectal linear array probe of 5.0 to 7.5 MHz frequencies (Filteau and Des Coteaux, 1998; Vyas et al., 2002; Ali and Fahmy, 2008; Kahn et al., 1990). Using the same probe pregnancy can be diagnosed with sufficient accuracy a little earlier (24-25 days) in the mare (Pycock, 2007). Recently, the use of a 3.5 MHz transcutaneous sector probe applied over the right flank has been suggested for pregnancy diagnosis in cows between 73 to 190 days of gestation. The fetal thoracic, abdominal and umbilical diameter was significantly associated with gestational age (Humman et al., 2009a, b, c). A trans rectal probe of 5.0 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., 2001b; Suguna et al., 2008). The diagnosis of pregnancy in the bitch requires a transabdominal probe (of frequency 3.5 to 5.0 MHz) to visualize pregnancy with accuracy from 25-30 days (Bondestam et al., 2008). The visualization of earlier pregnancy or the visualization of a non-pregnant bitches uterus necessitate the use of probes of higher frequency (7.5 to 10.0 MHz) as the uterus lie more closer to the skin. Color Doppler ultrasonography in the bitch can detect placental fetal circulation (Blanco et al., 2008) and in sheep pregnancy can be diagnosed with high accuracy at 76-90 days of gestation (Ganaie et al., 2009).

Doppler ultrasonography and A-mode probes are generally used in pigs for pregnancy diagnosis but B-mode probes with frequencies of 3.5 to 5.00 MHz can diagnose pregnancy with high accuracy between days 25-30 post mating (Almond and Woodard, 1997; Williams et al., 2008).

Radiography
To a limited extent radiography has been used for pregnancy diagnosis in the small ruminants (sheep and goat), the companion animals (dog and cat) and rarely in pigs. The technique is known to be good in evaluating fetal numbers in the bitch and cat, but is poor in evaluating fetal viability. 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 warrants its use in specialized cases. 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 (Grace et al., 1989; 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.

In bitches fetal skeletons are visible with high accuracy only by the sixth week of pregnancy although they may be sometimes visible as early as 23-25 days of gestation (Toal et al., 2005). The fetal skulls are visible by day 45 and the entire fetal skeleton is visible by the end of seventh week of gestation. The accuracy of radiographic diagnosis is dependent on the quality of radiograph obtained. Radiography is generally suggested for bitches and cats, whenever there is a doubt about the nature of the abdominal contents at or near whelping. The number and position of the fetuses can be detected easily by radiography at this time. Signs of fetal death as seen by radiography include the spalding sign, (which is the overlapping of the cranial bones), gas shadows in the fetal heart and stomach and tightly flexed spine (seen in fetuses died for long time) (Jackson, 2004).

The fetal skeletons begin to calcify only after the sixth week in sows and hence radiography should be performed only after this time for pregnancy diagnosis in sows (Rapic, 1961). Because pregnancy can be diagnosed with high accuracy with other methods, radiography is seldom performed for pregnancy diagnosis in sows.

Abdominal Ballottement

Abdominal palpation and abdominal ballottement of the fetus is possible to some extent in cows during late gestation (7 months onward). 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) has been suggested (Ott et al., 1981; Hulet,1972; Chauhan and Waziri, 1991;). Similarly bimanual palpation for pregnancy diagnosis (palpation of uterus through fingers in the rectum and lifting the abdomen) has been reported for small ruminants (Chauhan and Waziri, 1991; Kutty, 1999) however, both the methods are inaccurate and the first method is often invasive. 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.

In cattle abdominal ballottement is performed by placing the fist over the lower right abdominal wall and pushing it in an intermittent manner in a dorsal medial direction deeply. The fetus can be felt as a hard solid object floating in fluid. This is usually possible in lean cows after the 7th month of gestation. The fetal movements can be seen at the same place by careful visual observation however, the method is applicable too late in diagnosis. Abdominal palpation of pregnancy in bitches may be possible in small or medium sized bitches which are not too obese. Moreover, bitches often tense their abdominal wall and respire too fast making abdominal palpation often too difficult to perform. At about 30-35 days the accuracy is high (87%) and depends on the palpation of tense conceptual swellings (6 to 30 mm in diameter) within the uterine cornua but they must be differentiated from feces in the colon and the palpator must have expertise to differentiate or identify them correctly. The bitch’s foreparts must be slightly elevated. Beyond day 55 it is easy to diagnose pregnancy by the palpation of the fetuses. They may be palpable in the flank and also in the lower abdomen. With the availability of more effective methods like ultrasonography for early pregnancy diagnosis with accuracy the use of abdominal palpation has been reduced as the palpator is many a time confused.

Fetal echocardiography

To a limited extent fetal echocardiography had been used in the past to diagnose in cattle, sheep and mares, but with the advent of ultrasonography its use has been limited.

Vaginal electrical resistance

The conductivity of the vaginal mucous membranes changes at estrus due to increased hydration, increased blood supply and other changes. When measured by ohm meters the vaginal electrical resistance (VER) is low at estrus. Hence when VER is measured constantly animals returning to estrus can be identified and thus those probably becoming pregnant can be differentiated (Foote et al 1979; Mc Caughey, 1981; Gupta and Purohit, 2001; Meena et al., 2003) however the accuracy of such estimations in diagnosis of pregnancy is limited due to false positive results and the problem related with daily probing of animals.

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 limits the use of this technique as a means of pregnancy diagnosis in most animals.

**Laboratory tests for pregnancy diagnosis**

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. Unfortunately, none of the methods developed so far in animals are as accurate as the detection of hCG in pregnant human females. However, the research to develop commercial indirect methods continues because these methods are non invasive and the tests can be marketed to and performed by the dairy farmers. The currently available methods are briefly described.

**Progesterone hormone assay**

The corpus luteum formed on the ovary subsequent to ovulation produces progesterone for maintenance of pregnancy for a reasonable time period in some species and for entire gestation in other species like the cow, buffalo, goat and sow.

In normally cycling cows the CL is lysed because of the effects of prostaglandins from the uterus if the animal is not pregnant, and thus the progesterone level goes down. Therefore, low progesterone concentrations in maternal blood at 18 to 24 days post breeding can predict that the animal is non-pregnant and high progesterone gives an insight that probably the animal is pregnant. The specificity of progesterone tests conducted between 18 and 24 days post breeding have shown a specificity of around 98% (Zaied et al., 1979; Laing et al., 1980; Waldman, 1993; Gowan et al., 1982; Pennington et al 1985; Nebel et al., 1987) and is the easiest proven method for identifying non-pregnant (regularly cycling) animals post breeding. However, for the pregnant animals the accuracy of the test is low (75%) because of early embryonic death which alter the results. Commercially available ELISA, plasma or milk progesterone assay kits have not become popular due to their high cost and a low specificity. Non pregnant cows not returning to estrus and pregnant cows in which embryonic death occurs at a later time can both give false results. Likewise, in mares, sheep, goats, buffaloes, camels and sows assay of plasma or milk progesterone is not very accurate for diagnosis of pregnancy (Zarkawi, 1997; Kaul and Prakash, 1994; Sato, 1977; Fleming et al., 1990; Dionysius, 1991; Almond and Dial, 1986; Ellendorf et al., 1976; Abdel Rahim and El-Nazier, 1987). Since progesterone concentration in the peripheral blood of pregnant bitches is similar to those of non pregnant bitches, and since there is no placental progesterone produced in the pregnant bitch (Verstegen-Onclin and Verstegen, 2008) hence progesterone assay cannot be used to diagnose pregnancy in the bitch.

To a limited extent plasma progesterone has been used as a means of pregnancy diagnosis in the female camel in which species the CL is required for the entire gestation (Abdel Rahim and El-Nazier, 1987).

**Estrone sulfate**

One of the earliest written records of a urine-based pregnancy test can be found in an ancient Egyptian document. A papyrus described a test in which a woman who might be pregnant could urinate on wheat and barley seeds over the course of several days: “If the barley grows, it means a male child. If the wheat grows, it means a female child. If both do not grow, she will not bear at all.” Testing of this theory in 1963 found that 70 percent of the time, the urine of pregnant women did promote growth, while the urine of non-pregnant women and men did not. Scholars have identified this as perhaps the first test to detect a unique substance in the urine of pregnant women, and have speculated that elevated levels of estrogens in pregnant women’s urine may have been the key to its success.

A similar test of germination of wheat seeds when they are soaked in urine from pregnant cows which inhibits germination compared to urine from non-pregnant cows which stimulate germination has been described to be known as Punyakoti test (Nirmala et al., 2008). The differential germination is considered to be because of the presence or absence of estrogens in the urine. However, controlled studies using estrogen and progesterone failed to affect the germination rate of wheat seeds (Nirmala et al., 2008).

The estrone sulfate 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 appropriate day, at which detection of estrone sulfate detection is possible in the body fluids or secretions are mentioned in Table 2. 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 feces are the most easily collected specimens (Bamberg et al., 1991; Hermann et al., 2005). Commercial kits have been developed for pregnancy detection in mares by using on farm kits like Wee-Foal-Checker® or Equitest ES® which require urine or serum as the test material. These commercially available tests are recommended to be performed only after 120 days of gestation and specially suggested for miniature horses and donkeys in which pregnancy diagnosis by rectal palpation or ultrasonography is extremely difficult. The test procedures utilize an immunochromatographic procedure to measure concentrations of pregnancy specific steroids (estrogens) and manufacturer recommendations should be strictly followed for optimum results.

Chemical tests for pregnancy diagnosis
Most chemical tests reported in the past appear to be of historic importance only in current day pregnancy diagnostic procedures. Some of the chemical tests that utilize urinary estrogens, or other molecules as a basis of pregnancy diagnosis in domestic animals are described below:

**Cuboni test:**
This test was first developed by Cuboni (1934) and modified later (Galina and Cox, 1969). The test is performed in the mare for detection of pregnancy through assay of urinary conjugated estrogens. The test is performed as follows:

“To 15 ml of urine 3 ml of concentrated hydrochloric acid is added and heated in a water bath for 10 minutes and then cooled under a tap. To this 18 ml of benzene is added and shaken vigorously for half a minute. The supernatant (mainly benzene) is collected in another tube and 3 to 10 ml of concentrated sulfuric acid is added and the mixture heated in a water bath at 80°C for 5 minutes, and then allowed to cool. A positive (pregnant) test is indicated by the appearance of a dark, only green fluorescent color in the lower sulfuric acid layer and a negative (non-pregnant) is characterized by absence of fluorescent color and presence of a brownish color.”

The cuboni test is only effective beyond 150 days of gestation, and also predicts fetal viability.

Other chemical tests previously described for the detection of urinary estrogens in mares include the vaginal cornification, mouse or rat tests using ovariectomised female rats or mice, and the phenolsulphonic acid test. (Based on kober calorimetric test for estrogens) (Roberts, 1985; Benesch and Wright, 2001). In the mouse test, the serum or urine from pregnant mares when injected to ovariectomised mouse or rats would induce vaginal edema, appearance of cornified cells and mucus discharge due to presence of estrogens in the pregnant mare’s serum or urine (Allen and Doisey, 1924). The phenolsulphonic acid test utilizes ether extraction of urinary estrogens subsequent to removal of urinary pigments by hydrolysis and their conjugation with phenolsulphonic acid reagent after evaporation of the ether. The final reaction gives a pink to cherry red color if the urine is from pregnant mares (Mayer, 1944; Benesch and Wright, 2001). The test is lengthy and cumbersome and requires special precaution in processing which has turned out this test of historic importance only. These tests are 70-80% accurate when performed between the 120 to 250 days of pregnancy.

Another test described previously include the mucin test in which the vaginal mucus from a pregnant mare showed dark staining columnar epithelial cells (pregnancy cells) (Kurosawa, 1931) and known to have an efficacy of 94% from day 70 to end of gestation (Miller and Day, 1938; Day and Miller, 1940) however, these old tests have now been replaced because of availability of better diagnostic, highly specific radio immunoassays and enzyme immunoassays.

Barium chloride test: A test has been described for pregnancy diagnosis in the bovine species commonly known as the Barium chloride test. To 5 ml of urine from cows a few drops of 1% barium chloride is added and warmed slightly. In non pregnant cows a white precipitate is formed, whereas, in pregnant animals the urine remains clear (Temblador and Landa, 1971). The accuracy of the test was described to be 70-95% (Maslov and Smirnov, 1965; Elpakov and Cyganok, 1966; Akmadeev and Vasilyev, 1967) from 15 to 210 days of pregnancy; however, a later study (Kavani, 1976) noticed only a low accuracy (64%) with a high occurrence of false positive and false negative results. In camels the test was considered to be 85% accurate between days 50-90 of pregnancy (Banerjee, 1974). By and large the validity of such a test continues to be controversial.

Some other previously described tests for pregnancy diagnosis in cows include two tests on milk; i) milk alchohol coagulation test: in this test there is coagulation of milk from pregnant cows when mixed with equal quantities of alchohol and allowed to stand for 1-3 hours (Linkes, 1930; Rutz, 1932; Stancev and Angelov, 1966; Kavani, 1976). ii) copper sulfate test: 1 mL of milk when mixed with a few drops of 3% copper sulfate coagulates if the animal is pregnant (Temblador and Acosta, 1971; Kavani, 1976). The accuracy of these tests is considered to be low (52.0 to 64.2%) and many a times the tests are inconclusive and
Several biologic tests were developed for the detection of eCG including the Aschheim Zondek Test, the Friedman test (rabbit test) or the Frog or toad test. 

Aschheim Zondek test

Aschheim and Zondek described a test (known as the A-Z test) which identified the presence of hCG in human urine. To test for pregnancy, a woman's urine was injected into an immature rat or mouse. If the subject was not pregnant, there would be no reaction. In the case of pregnancy, the rat would show an estrous reaction (be in heat) despite its immaturity. This test implied that during pregnancy there was an increased production of the hormone. A similar test in mares has been described wherein serum from mares is injected to mice and the results are read later (Miller and Day, 1938). The serum (0.5 ml SC daily for 2 to 4 days or 5 ml intraperitoneally) from test mare is injected to 2 to 3 rats (22 days of age) and rats are killed (72 hr when injected intraperitoneally and 96 to 120 h later when injected subcutaneously) and a positive test is indicated by the presence of multiple corpora haemorrhagica on the ovaries and uterine edema. The test was considered to be 90 percent accurate when performed between the 60 to 100 days of pregnancy in the mare. The same test performed in camels revealed no changes in the mice (Banerjee, 1974; El-Azab and Musa, 2007) due to lack of any gonadotropic molecule.

Friedman Rabbit test

Serum from test mare is injected (2 ml given IV) to rabbits (14 to 20 weeks age) kept in isolation and laparotomy performed 24 h later. A positive test is indicated by the presence of corpus haemorrhagicum and uterine edema (Chicchini and Chiacchiarini, 1963).

Toad test

Toads like Bufo valliceps or frog like Rana Pipiens are used in this test. The basis of this test is the concept that the sperm cells are emitted by toads/frogs only when stimulated with female frogs or gonadotrophins. Two male toads are taken and their cloaca is wiped with normal saline and examined for presence of spermatozoa. If no sperms are present the cloaca is cleaned and 1 ml of test serum from a mare is injected into the dorsal lymph sac of 2 male toads thrice at an interval of 1 hour. The cloaca is examined for the presence of the sperms. A positive test is indicated by the presence of sperms in the cloaca within 1 to 6 hours after the last injection (Cowie, 1948; Neto, 1949) Similar test depicting the presence of a gametokinetic substance in the feces of dairy cows, buffalo, goat and sheep has been depicted which also initiate a similar response in the cloaca of the frog Rana Pipiens (Bhaduri and Bardhan, 1949; Kavani, 1976). Moreover, no specific molecules were determined in these studies and therefore their use in current diagnostic methodologies is limited and usually of historic importance. Similar to these tests a previously described test documented the appearance of deep yellow to orange coloration of cervical mucus from non-pregnant cows when boiled with 10-15% NaOH whereas cervical mucus (which is difficult to obtain from pregnant cows) from pregnant cows evidenced a pale yellow or colorless state (Sokolovakaya et al., 1959; Williams, 1964; Kavani, 1976).

Costa’s test: This test was developed by Costa (1927) for testing of pregnancy in human females and is based upon sedimentation of haematin in the presence of a solution of novocaine. To 1.5 mL of 2% novocaine 3 drops of blood are added and then 5% solution of sodium citrate is added. The mixture is centrifuged and 1 drop of formalin is added. In pregnancy grey or grayish yellow color or precipitate appears within 15 minutes. The test was experimented in cows and the test was known to be positive after 38 days of insemination with 65.3% accuracy in cows (Krissa, 1934, Bhattarcharya, 1967; Kavani, 1976) and inaccurate in camels (Banerjee, 1974).

Kosjakovs test: This test apprehends that the sulfur content of hair in pregnant animals is increased (Kosjakov, 1929). Hair from animals under test are digested with 10% potassium hydroxide and boiled with 2 ml of distilled water and 1% methylene blue and a few drops of 4% hydrochloric acid are added. The blue color disappears in pregnant animals due to increased sulfur. The basis of the test was not verified in subsequent research and hence the test is now obsolete.

**Assay of gonadotrophins**

The human female secretes the gonadotropin hCG which is present in sufficient quantities in the urine of pregnant women and many simplified tests have been developed to detect this molecule in urine for an easy pregnancy diagnosis in women. The only animal species that secrete sufficient quantities of gonadotrophins that can be used as a marker molecule for pregnancy diagnosis is the equine (Cowie and Hart, 1930). Endometrial cups form as early as day 35 and secrete the equine chorionic gonadotrophin (eCG) which can then be detected from serum and urine (Roser and Lofstedt, 1989) of pregnant equine females. The eCG continues to be secreted from day 40 to 120 days of pregnancy and is the basis of previously described biologic tests and the currently available on farm tests.

**Biologic tests**
Bhaduri, 1951; Banik and Reineke, 1955) although the validity of the toad test in species other than the mare remains doubtful.

The above mentioned older methods have now been replaced by newer methods including radioimmunoassay (Nett et al. 1975), radioreceptor assay (Fay and Douglas, 1982), haemagglutination inhibition test (Berthelot et al. 1987), ELISA (Squires et al 1983) and direct latex agglutination tests (Decoster et al 1980). Commercially available kits are in use at many places for these assays (Envet Services, Omaha NE). Pregnamare® is one such test, which can be performed on blood between day 40 and 100 of pregnancy, however, false results may be obtained if fetal death occurs after formation of the endometrial cups.

**On farm tests**

Some commercial kits are currently available which can detect the presence of eCG in blood of mares between 40-100 days of pregnancy with 96-98% accuracy. Pregnamare is such a kit which requires 5 drops of blood collected from the muzzle by using the lancet provided with the kit. This color change test requires about one hour completing.

**Other methods**

Some of other methods described previously include onset of estrus by injections of estradiol valerate (2mg) at day 9-12 of service in cows (Jochle and Schilling, 1965; Kavani, 1976) and day 17-18 in sows (Laerum et al., 1974). The potential dangers of injecting estrogens to pregnant cows have refrained from the large scale use of these tests. Some years ago, a pregnancy diagnosis based in determining differences in cervical mucus using nuclear magnetic resonance was used (Merilan, 1983). This test looked promising at that time but no subsequent information is available.

**Milk ejection by low dose prostaglandin**

A method tested some years ago was the injection of low dose prostaglandin F2 alpha (non-luteolytic dose) in animals two weeks after breeding resulting into milk ejection. The animals detected further as pregnant showed an increase in the pressure in the milk ejection and alveolar milk volume collected by a teat probe in comparison with the non-pregnant cows (Labussiere et al., 1982; Prakash et al., 1996) and ewes (Labussiere et al., 1983; Labussiere et al., 1988). The intrajugular administration of a subluteolytic dose of PGF2 alpha induces a large increase in intramammary pressure when given during the luteal phase and this is directly correlated to the plasma progesterone profiles (Labussiere et al., 1988). However, due to potential dangers of inducing luteolysis by accidental over dosage, the use of this technique of pregnancy diagnosis, could not gain wide popularity.

**Pregnancy associated glycoproteins (PAG)**

Pregnancy specific proteins are known to be produced in various ruminant species including cattle, buffalo, sheep and goats (Humblot et al., 1990; Karen et al., 2003; Karen et al., 2007). Two pregnancy specific proteins (PSP) A and B have been isolated from bovine fetal membrane extracts (Butler et al., 1982). Of these PSP-A was identified as a a-fetoprotein and PSP-B was found to be specific to the placenta. These molecules appear in the maternal circulation and can be determined with accuracy from 29 to 30 days post breeding. The PAG continues to be existent in maternal blood for the entire pregnancy and up to 100 days post partum. The assay involves radioimmunoassay on serum. The sensitivity and specificity of PSPB based on RIA is known to be 92.0% and 82.6 to 91.9% from 29 to 30 days post insemination (Szenci et al., 1998). More recently simple ELISA techniques have been developed that detect the PAG molecule in the serum of cows (Breed et al., 2009; Green et al., 2005; Green et al., 2009; Silva et al., 2007). The limitations to the wide spread use of this test is non availability of the protein in milk or urine, presence of PAG up to 100 days post partum (which interfere with subsequent detections) and the non availability of cow side commercially available kits for its detection. Recently however, the existence of PAG has been documented in bovine milk (Gajewski et al., 2008).

**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. 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). The developing embryo bears antigens foreign to the mother; hence immune rejection of the early embryo may occur. An immunosuppressive early pregnancy factor (EPF) appears as early as 6 to 48 h of mating which functions to suppress the maternal immune response thereby allowing for pregnancy to proceed (Shaw and Morton, 1980). In cattle significant differences in rosette inhibition titer were observed between pregnant and non pregnant cows on day 13-16 and 25 post breeding (Sakonju et al., 1993), suggesting that measurement of EPF activity may be useful as an indirect method of pregnancy diagnosis. A commercially marketed kit is available in the US (ECF test, concepto Diagnostics Knoxwille, TN) however its reliability is known to be poor (Adams and Jardon, 1999; Des Coteaux et al., 2002) and need to
be substantially improved.

**Relaxin assay**

Relaxin can be determined in the peripheral circulation of pregnant bitches at 20-30 days of gestation, whereas it is absent in non-pregnant bitches at all stages of the reproductive cycle. (Steinetz et al., 1989). This relaxin is known to be produced by the placenta in the bitch and cat and is thought to contribute to its maintenance by inhibiting uterine activity. In the domestic dog it has been established as a pregnancy-specific hormone (Steinetz et al., 1987, 1989). The site of synthesis in the bitch has been elucidated (Tsutsui and Stewart, 1991; Klonisch et al., 1999) and primarily ascribed to the placenta, although the hormone can also be traced in the ovary and uterus. These latter tissues may be areas influenced by the paracrine deposition of relaxin. A clinical study of domestic dogs using the commercially available canine relaxin enzyme-linked immunoassay (ELISA) (ReproCHEK, @a Synbiotics Corporation, San Diego, CA, USA) reported detection of the hormone in maternal peripheral blood as early as 25 days after ovulation (Buff et al., 2001). In the cat it appears during the third week of pregnancy, with concentrations declining just before parturition (Stewart and Stabenfeldt, 1985). The molecule has also been detected and used successfully for pregnancy diagnosis in the non domestic species (Carlson and Gese, 2007; Bauman et al., 2008).

**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 in the sow (Done and Heard, 1968; Morton and Rankin, 1969) and to a limited extent in sheep. (Richardson, 1972). The accuracy of this method between 18 and 22 days is 97% and 94% for the diagnosis of pregnancy and non-pregnancy respectively in the sow (Mc Caughey, 1979). 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. 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 sow the use of vaginal biopsy for pregnancy diagnosis has been reduced.

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### Table 1: Ultrasonographic features of early pregnancy in various species

<table>
<thead>
<tr>
<th>Sonographic Structure appearance (days post mating)</th>
<th>Cow</th>
<th>Buffalo</th>
<th>Mare</th>
<th>Sheep/Goat</th>
<th>Sow</th>
<th>Bitch</th>
<th>Camel</th>
<th>Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal fluid</td>
<td>18-20</td>
<td>18-22 days, 5th week</td>
<td>10-16</td>
<td>20-25</td>
<td>18-20</td>
<td>18-20</td>
<td>17-18</td>
<td>10-16</td>
</tr>
<tr>
<td>Fetal Heart beat</td>
<td>24</td>
<td>30</td>
<td>24-25</td>
<td>21-23</td>
<td>–</td>
<td>24</td>
<td>28-30</td>
<td>16-18</td>
</tr>
<tr>
<td>Cotyledons/Allantois</td>
<td>35-40</td>
<td>30-35</td>
<td>20-22</td>
<td>40-50</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>25</td>
</tr>
<tr>
<td>Fetal bones fetal buds</td>
<td>57-60</td>
<td>–</td>
<td>–</td>
<td>70</td>
<td>–</td>
<td>42-50</td>
<td>40 days</td>
<td>30-33</td>
</tr>
<tr>
<td>Fetal sex determination</td>
<td>57-60</td>
<td>10-18 weeks</td>
<td>60-70</td>
<td>60-90</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>38-43</td>
</tr>
<tr>
<td>Fetal movement</td>
<td>42-50</td>
<td>47-51</td>
<td>40-45</td>
<td>–</td>
<td>60</td>
<td>–</td>
<td>–</td>
<td>30-34</td>
</tr>
</tbody>
</table>
Table 2 Appearance of estrogen in domestic animals during pregnancy

<table>
<thead>
<tr>
<th>Species</th>
<th>Day of detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mare</td>
<td>Maternal estrogen high after 60 days Conjugated urinary estrogens high after 150 days</td>
<td>Sist et al. (1987); Cox (1971); Bhavnani and Woolever, 1978</td>
</tr>
<tr>
<td>Cow</td>
<td>Day 100 of gestation</td>
<td>Hamon et al. (1981); Robertson et al., 1978</td>
</tr>
<tr>
<td>Sows</td>
<td>Rise start at 20 days peak at 25-30 days followed by a decline at 45 days and again a rise at 70-80 days to term</td>
<td>Cunningham, 1982 Seren et al.(1983) Robertson et al.(1978); Gutherie and Deaver, 1979</td>
</tr>
<tr>
<td>Bitch</td>
<td>Slightly increased at implantation and remain constantly high for rest of gestation and decline 2 days prepartum</td>
<td>Concannon et al. (1975)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Detectable by day 70; rise thereafter till 2 days prepartum</td>
<td>Illera et al., 2000; Worsefold et al., 1986</td>
</tr>
<tr>
<td>Buffalo</td>
<td>Appear at day 150 of gestation in the serum</td>
<td>Prakash &amp; Madan (1993); Kamonpatana, 1984</td>
</tr>
<tr>
<td>Camel</td>
<td>Increase start at day 50 and peak from day 90-300</td>
<td>Skidmore et al., 1996</td>
</tr>
</tbody>
</table>
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