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Laboratory and imaging techniques for pregnancy diagnosis in animals

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Abstract
An accurate and early diagnosis of pregnancy is essential for better reproductive management in livestock and wild animals. Correct pregnancy diagnosis shorten the calving interval through early identification and maintain the animals dry period on regular time. In the absence of early and accurate detection of pregnancy important problems like high calving interval, late puberty, and high incidence of anestrous aggravates. There are several methods of pregnancy diagnosis in the domestic as well as wild animals and classified as visual, clinical and laboratory methods, yet there are limitations due to their sensitivity, accuracy, specificity, speed, and ease of performing the test. Laboratory methods of pregnancy diagnosis used in past and developed in recent years provided the results accurately. The advancement of molecular techniques like proteomics and their applications in animal research has given a new hope to look for pregnancy biomarker molecules in these animals. The present review provides a deep insight about the methods of pregnancy diagnosis in different species of animals while assessing the usefulness of the modern technology and discusses the future prospects.

Keywords: Pregnancy diagnosis, laboratory methods, immunological assays, chemical and biological

1. Introduction
Diagnosis of pregnancy (Cyesiognosis) in all species of animals needs to be done at the correct and appropriate time for giving proper care to the animals and to prevent economic losses. Accurate early detection of pregnant and nonpregnant cows is an essential factor for optimizing reproductive performance in dairy cattle [1]. It is equally important for other species of animal, be it domestic or wild species. In dairy cattle, pregnancy diagnosis is an important tool to measure the success of a reproductive management [1]. In mares it is utmost important to diagnose pregnancy in an early stage for the proper management of animal round the year. In case of wild animals non-invasive monitoring of hormones using feces has become a vital tool for reproductive management and reliable pregnancy diagnosis [2]. Lack of early pregnancy diagnosis create huge problem for farmers as well as for livestock owners [3]. Farmers can plan some alternative measure for the animal if animal will be pregnant or non pregnant. In some case pregnancy is not desirable especially for pet animals so for that cases it can be terminated early if it gets detected. There are several methods to diagnose pregnancy in animals but due to specificity and reliability of test there is possibility of false negative and false positive of the result. Moreover laboratory and imaging methods are more reliable for pregnancy diagnosis in all animal species [4]. This review details the important and salient tests used in the various laboratories as well as the imaging technique used for pregnancy diagnosis.
The importance of diagnosing pregnancy early lies in

a) Identifying non-pregnant animals soon after mating or insemination so that production time lost from infertility may be reduced by appropriate treatment or culling
b) Certifying animals for sale or insurance purposes
c) Reducing waste of time and money in breeding programs using expensive hormonal techniques
d) Assisting in the economic management of livestock and
e) Planning captive breeding programs
f) To reduce economic losses and make farm animal management profitable

2. Pregnancy diagnosis in animals can be done by various techniques as listed below
a) Visual method: Non return to estrus, increase in abdomen size, development of udder (heifers), cocking of tail (Camel)

b) Clinical methods: Recto-genital palpation, Abdominal ballottement, Fetal echocardiography, Laparoscopy
c) Laboratory methods:
   1. Immunological and Hormonal assays: Assay of Progesterone, Estrogen, PMSG, PAG, EPF, IFN-τ, Prolactin, MicroRNA, CEP, Relaxin and PGFM
   2. Chemical tests: Cuboni test, Phenolsulphonic acid test, Mucin test, Barium Chloride test, Costa's test, Germination inhibition test.
   3. Biological tests: Aschiem Zondek test, Friedman rabbit test, Toad test and Mouse test
d) Imaging techniques: Ultrasonography and Radiography

3. Hormonal and immunological methods

3.1 Progesterone (P4) hormone assay

Progesterone is one of the most important hormones of female reproduction and is known as pregnancy hormone, responsible for maintaining pregnancy. In a non-pregnant cyclic animal, the development of the corpus luteum causes the progesterone levels to increase in luteal phase (metestrus and diestrus from 3rd/4th day to 17th/18th day of estrous cycle) and the regression of corpus luteum causes a gradual decrease in follicular phase (proestrus and estrus from 17th/18th day to 2nd/3rd day of estrous cycle). This pattern is cyclic in the non-pregnant cycling animal. In case of pregnant animals progesterone level remains elevated throughout the pregnancy (until parturition) causing the absence of cyclic pattern. Progesterone is an example of non- pregnancy specific diagnosis method [5].

Species: Bovine, Caprine, Ovine, Equine and Swine
Principle: Elevated progesterone levels in pregnant animals are maintained by either corpus luteum or placenta (Sheep, Horse). Therefore the determination of the progesterone (P4) concentration in milk or plasma (by RIA or ELISA) can be a good indicator of pregnancy in animals.

Table 1: Normal levels of P4 in different animal species

Species	Non pregnant (ng/ml)	Pregnant (ng/ml)
Cow [6]	<2	>5
Doe [7]	<1	>2
Ewe [8]	<1	>2
Sow [9]	<4	>5
Mare [10]	<1	>4

Procedure: After an insemination or mating the milk sample is collected on the completion of one estrous cycle length in the respective species e.g. 22 to 24 days in cattle and buffalo, 16 to 18 days in sheep, 18 to 21 days in goat, 16 to 22 days in horse and 21 days in pigs. The progesterone level is determined in the collected sample by RIA or ELISA.

Day of detection: Pregnancy can be detected as early as 22-24 days post insemination in Cattle, Buffalo [11, 12].

Accuracy: Pregnancy prediction- 75 to 90%, non-pregnancy - 100%.

Disadvantages: False positive pregnancy determination may occur due to high progesterone concentration detected in many cases like severe uterine infection, some cystic ovarian conditions and in irregular estrous cycle. This test is unreliable in the horse because of prolonged diestrus (high P4) and also in cases of early embryonic death.

3.2 Estrone sulfate hormone assay

Estrone sulfate is a conjugated steroid product of estrone, produced by the conceptus and can be measured in maternal plasma, milk, urine or feces in all farm species. Detectable in the plasma earlier in the sow (day 20), mare (day 60), goat and sheep (day 40-50) and cow (day 72) [13]. Estrone sulfate is a pregnancy specific marker [5]. Also used for pregnancy detection in non-domestic animals like gorillas, orang-utans, balsams and wild felids by using its fecal and urine samples [14].

Species: Equine, Swine, Bovine, Caprine, Ovine
Principle: Estrone sulfate produced by conceptus can be detected by RIA or ELISA combined with thin layer chromatography.

Table 2: Days of detection of pregnancy by estrone sulfate assay

Species	Days of detection after mating
Bovine	72nd day onwards
Caprine	50th day onwards
Ovine	50th day onwards
Swine	26th day onwards
Equine	60th day onwards

Accuracy: For pregnancy determination- 90-95%. False-positive may occur with haemolysed serum samples. False negative may occur if samples are collected before 50 days of gestation (Equine, Bovine).

Advantage
- It helps in determining the viability of the fetus.
- Concentration of estrone sulphate has been correlated to foetal membranes [15].

Disadvantage: Compared to progesterone determination, detection of estrone sulphate is done later in the pregnancy period.

Table 3: Normal Estrogen levels in different animal species

Species	Non pregnant (pg/ml)	Pregnant (pg/ml)
Cow [16]	20-25	<50 (at 2 month)
Doe [17]	20-22	5-10
Ewe [18]	10-15	5-10
Sow [9]	<500	>500
Mare [19]	25-40 ng/ml	15-20 ng/ml

3.3. Pregnant Mare Serum Gonadotropin (or) Equine Chorionic Gonadotropin Assay

Principle: Equine chorionic gonadotropin (eCG or PMSG) produced by uterine endometrial cups appears in the blood of mares as early as 40 days following conception and its detection helps in early diagnosis of pregnancy. PMSG (or) eCG, when present in the blood sample, prevents agglutination of sensitized sheep red cells by anti-eCG. It can also be detected by RIA, Latex agglutination test, polystrenic agglutination test, ELISA etc.

Species: Equine

Procedure

Mare Serum + anti eCG with indicator
→ Agglutination inhibited (Pregnant)
→ Agglutination occurs (non pregnant)

Day of detection: 40th day onwards up to 120th day when the levels starts to fall [20].

Accuracy: Pregnancy detection- 90% accurate, false negative 5% and false positive 10%.

Disadvantage: If the fetus dies during period of 40-120 days, false positive results are obtained with high plasma eCG levels.

Normal Levels: 10-100 IU/ml

3.4 Pregnancy associated glycoproteins (PAG)

Pregnancy-associated glycoproteins (PAG) also known by other names like pregnancy specific protein B (PSPB) or pregnancy-specific protein 60 (PSP-60), were first described as placental antigens that were also present in the blood serum of the mother soon after implantation. These glycoproteins are synthesized in the mono and binucleate cells of the ruminant's trophectoderm and formed in several ruminant species viz. cattle, buffalo, sheep and goats. Two pregnancy specific proteins (PSP) A and B have been isolated from bovine foetal membrane extracts. The PSP-A was recognized as alpha-fetoprotein and PSP-B was found to be specific to the placenta. PSP-B is mostly used for pregnancy detection in bovines. Lopez-Gatius F (2007) [21] reported that as milk production increases in high producing cows, the PAG concentration decreases (negative correlation). Further Pohler KG (2016) [22] reported that as parity increases the PAG concentration decreased linearly till the 3rd to 4th parity.

Species: Bovine
Principle: Identification of proteins specific to pregnancy by RIA, ELISA.

Day of detection: From 28th day of post mating or post insemination.

Accuracy: Pregnancy determination- 80-90% accurate.
Disadvantage: Pregnancy specific proteins can be found in blood during entire gestation as well as period up to 100 days after post parturition which interferes with the results.

Normal Levels: 1.0 ng/ml is normal value to discriminate between pregnant and non-pregnant animal (buffaloes) [23].

3.5 Early pregnancy factor

Early pregnancy factor (EPF) is a pregnancy associated immunosuppressive protein, which has been detected in sera of pregnant animals by rosette inhibition test (RIT)

Species: Bovine, Caprine, Ovine, Swine
Principle: Detection of EPF by its immunosuppressive ability. Rosettes are formed when lymphocytes are treated with heterologous RBC but when lymphocytes are treated with antilymphocyte serum this rosette formation is inhibited. When the lymphocytes are pre-treated with EPF containing serum, this rosette formation is greatly inhibited thereby decreasing the quantity of anti-lymphocyte serum to produce the same inhibitory effect.

Day of detection: The presence of EPF in serum provides the earliest indication that fertilization has taken place.

Table 4: Occurrence of EPF in different species

Hours after fertile mating	Species
4-6	Rat [24]
24-48	Cattle [25]
	Sheep [26]
	Mare [27]
	Pig [28, 29]
	Human [30]

Advantage: Used for the early detection of pregnancy or early embryonic mortality.

Disadvantage: EPF is pregnancy-dependent protein rather than pregnancy specific, so the levels are tend to be high in case of tumours associated with ovaries.

Normal Levels: EPF concentration should be at least 100pg/ml for detection by rosette inhibition test.

3.6 Interferon tau

Interferon tau (IFN-τ) is first produced by the conceptus between 12-13 days in sheep and 14–16 days in cattle [31] after pregnancy is established. In ruminants, once pregnancy is established, estrogen and oxytocin receptors are inhibited to prevent the release of PGF2α (luteolysin)

Species: Ovine, Bovine
Principle: Interferon tau is indirectly estimated by identifying the interferon stimulated genes(ISG) namely Mx2 and ISG 15 in Ovine and Oas1 (Oligoadenylate Synthetase) in Bovine by Reverse Transcriptase Polymerase Chain Reaction (RTPCR) during pregnancy.

Day of detection: Sheep: 12-13 days and Cattle: 14-16 days after insemination.

Disadvantage: Gene expression may alter under many pathological conditions. ISG are not unique to pregnancy [32].

Normal Levels: Since it depends on the value of gene expression, normal levels are purely comparative.

3.7 Prolactin assay

Prolactin is a polypeptide secreted by the lactotroph cells of anterior pituitary gland. Its main role is in stimulating milk synthesis. It tends to rise after 5-7 months of gestation and remains elevated until parturition.

Species: Elephant (*Elephas maximus*)
Principle: Prolactin levels (detected by RIA/ELISA) tend to be elevated from the last part of first trimester and maintained until few weeks prior to parturition in pregnant elephant. Gestation length in elephant is 22 month.

Day of detection: From 7th month of pregnancy [33].
Normal Levels: Non pregnant animal have 6.9 ng/ml, while pregnant animal have 50 ng/ml [34].

3.8 MicroRNA as biomarkers in pregnancy

Various pregnancy diagnosis tools viz. rectal palpation, ultrasonography, milk progesterone test, and pregnancy-associated glycoproteins have been widely utilized in recent times. However, there are limitations related to sensitivity and specificity of these tests that could lead to false positive and

negative results. Most importantly, most of these techniques work better after day 21 of post insemination. Hence, development of non-invasive biomarkers that could be utilized as indicators of early pregnancy in livestock could be proposed as an alternative tool. For this purpose in recent time microRNAs (miRNAs) which is a subclass of small non-coding RNAs and is 18-22 nucleotides long molecules emerged as key posttranscriptional regulators of gene expression. Circulatory form of miRNAs used as attractive tools in predicting the pregnancy in livestock animals.

Species: Equine [35], Ovine [36, 37] Bovine [38]
Principle: Pregnancy specific miRNA are amplified by using RT-PCR, which can be detected as a marker of pregnancy.

Day of detection: 16 days post- fertilization [5]
Normal Levels: Since it depends on the value of gene expression, normal levels are purely comparative.

3.9 Ceruloplasmin early pregnancy assay (CEP assay)
Acute phase protein ceruloplasmin increases in urine of giant pandas in response to pregnancy which can be used as potential biomarker of pregnancy.

Species: Giant panda (*Ailuropoda melanoleuca*)
Principle: Ceruloplasmin tends to increase 19 fold during pregnancy which is excreted in urine and can be detected by calorimetric methods. The level is not elevated in case of pseudo pregnant animals.

Day of detection: Active urinary ceruloplasmin was elevated from first week of pregnancy up to 20-24 days prior to parturition [39].

Normal Levels: Normal level of ceruloplasmin is 20-50 µg/dl in non pregnant animal [39]. Gestation period in Giant panda is 95-160 days.

3.10 Relaxin assay
Relaxin a water soluble polypeptide hormone is mainly produced from placenta (Canine, Feline) and corpus luteum. It inhibits uterine myometrial contraction, augment uterine growth to accommodate the growing foetuses increases the flexibility of the pelvic girdle and the distensibility of the uterine cervix in preparation for parturition. Temporal changes in circulating concentrations of immuno reactive relaxin during pregnancy and the peri-partum period appear to be species specific.

Species: Canine, feline [40, 41]
Principle: Relaxin appears in the peripheral circulation in pregnant animals whereas it is not the case in non-pregnant and pseudo pregnant animals. The circulating relaxin can be detected by using ELISA or RIA.

Day of Detection

Species	Days of detection (Post mating)
Domestic cat	21-28
Domestic dog	21-25
Coyotes	25
Wolves, Foxes and Leopard	25-28
Rhesus monkey	21

Normal Levels: 5.63 ng/ml (in nonpregnant) and 13.33 ng/ml (in 28 day pregnant animals) [42].

3.11 PGFM (13, 14-dihydro-15-keto-PGF$_{2\alpha}$) assay
PGFM or 13, 14-dihydro-15-keto-PGF$_{2\alpha}$ is a metabolite of PGF2α and acts as an indicator of pregnancy in various feline species. PGFM is detectable in urine and feces of canine and feline species. In carnivores especially in large felids PGFM has been applied as a useful analytical marker of pregnancy and its concentration in urine and feces rises prior to parturition in large feline. For its identification in feces High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LCMS) techniques has been applied. The importance of detection of PGFM in the urine or feces is to differentiate the true pregnancy from pseudopregnancy in domestic as well as non domestic felids.

Species: Feline (wild), Asiatic Lion, Bengal Tigers, Jaguars.

Principles: PGFM are usually found in urine/feces only prior to parturition but in case of felines they are elevated and maintained at detectable levels in urine/feces which can be detected by Enzyme Immuno-Assay and High Performance Liquid Chromatography.

Day of detection: 56 days of gestation in Large Feline [43].

Advantage: PGFM as a clear signal differentiating between pregnant and pseudo pregnant luteal cycles in domestic and non-domestic felid species.High levels allow pregnancy diagnosis without knowledge of mating time in free ranging animals [44].

Normal Levels: Basal concentrations of PGFM (830-1100 ng/g). Levels increase from week 8 onwards (1512 ng/g) until parturition (4987 ng/g) [45].

4. Chemical Tests
4.1 Cuboni Test
Species: Equines
Initially developed by Cuboni E (1934) [46]. The test is performed in the mare for pregnancy diagnosis through assay of conjugated oestrogens present in urine of pregnant mares.

Principle: Fluorescence is the characteristic of natural oestrogens which is used to detect the oestrogens (secreted by foetus) in the urine.

Day of detection: 150 days of gestation in mare [47].
Accuracy: 90%
Procedure: Add 3 ml of conc. HCl to 15 ml urine. Heat in water bath for 10 minutes and then add 18 ml Benzene. Shake vigorously for 30 second and separate the supernatant. Add 3 ml-10 ml conc. H_2SO_4 to the supernatant. Heat the content in water bath at 80ºC for 5 minutes. After cooling see it under fluorescent light. Presence of green fluorescence is positive for pregnancy. Brown colour without fluorescence indicates negative result.

4.2 Phenolsulphonic acid test / Kober's colour test
Kober (1931) was first to suggest colorimetric test for urinary estrogen. This test as modified by Pincus G (1936) [48] is used for identification of natural estrogen by forming conjugated coloured end product which could be detected by colorimeter. It is a two phase reaction which can also be used for

quantifying the estrogens present.

Species: Equine

Principle: Estrogen with phenolsulphonic acid reagent forms a conjugated end product which gives pink or cherry red colour.

Day of detection: 150 to 250 days of gestation.
Accuracy: 70 to 80%.
Procedure: Dried extract is added to 2 cc phenolsulphonic acid. On heating for 10 minute at boiling temperature a yellowish brown solution appears and then placed in ice water for 1 minute. Make it to 4 cc with 5% H_2SO_4 then cool in ice for a minute. Thoroughly mix, wait for 10 minutes for maximum colour to develop then read at 510-512 nm wavelengths. Appearance of pink or cherry red colour indicates positive result [48, 49, 50]. Non appearance of pink or cherry red colour indicates negative result.

4.3 Mucin test/ Kurosawa test

Principle: Dark staining columnar epithelial cells [51] (Pregnancy cells) can be identified by microscopic examination in the vaginal mucus of pregnant mare.

Species: Equine

Day of detection: From 70 days of gestation to end of gestation [52].

Accuracy: 94%

4.4 Barium chloride test

Species: Bovine, Swine and Camelidae.

Day of detection: Bovine: 15-215 days of gestation (accuracy 65-70 %). Camel: 50-90 days of gestation (accuracy 85 %), [53, 54, 55].

Procedure: Add a few drops of 1% $BaCl_2$ to 5 ml of urine and slightly warm it. Formation of white precipitate indicates negative test. If no precipitate forms and urine is clear it indicates a positive result.

4.5 Costa's test

Developed by Costa in 1927 for detection of pregnancy in human females.
Species: Bovine

Principle: Sedimentation of haematin in the presence of a solution of novocaine which gives grey-bluish colour precipitate.

Day of detection: 38 days post- insemination in cow [56].

Accuracy: 50-60%

Procedure: Add 1.5 ml of 2% Novocain to 3 drops of blood. Add 1 ml of 5% sodium citrate solution. Centrifuge at 1000-1500 rpm and add 1 drop of formalin. Formation of grey-bluish yellow colour precipitate indicates positive for pregnancy.

4.6 Germination inhibition test

The seed germination inhibition test is recognized as a door step technology to the farmers that can be done at farmers houses by the farmers since it require inexpensive materials and does not require special skills, it is a simple, farmer friendly and noninvasive method.

Species: Cattle [57], Goat, Sheep [58].

Principle: Seed germination inhibition caused by urine of animal indicates the pregnancy state of animals. Hormone metabolites excreted through urine of pregnant animal might affect the seed germination. Concentration of a plant hormone "Abscisic acid" is higher in the urine of pregnant animals (170.62 nmole/ml of urine) than non-pregnant animals (74.46nmole/ml) [59].

Day of detection: Cattle: 2 month of pregnancy. Sheep and Goat: Two to three weeks of pregnancy

Procedure: Collect the urine sample from the animals suspected to be pregnant. Dilute the urine into 1:4 dilutions with distilled water. Take 15 wheat seeds over a blotting paper in a sterile petridish. Add 15 ml of diluted urine over the seeds in petridish and cover it to avoid evaporation. Keep it undisturbed for three days. After three days examine the seeds for germination inhibition percentage and shoot length.

Result: Mean germination inhibition percent and reduced shoot length in positive group is indicative of pregnancy state.

4.7 Other chemical test's used

Table 5: Other chemical tests to diagnose pregnancy

Test	Species	Procedure	Positive for pregnancy
Copper sulphate milk test [60]	Bovine	1 ml of milk + few drops of $CuSO_4$	Coagulation occurs
Sodium hydroxide- mucus test [55]	Bovine	5 ml of 10% NaOH + Cervical mucus, Then boil it	Appearance of orange colour
Copper sulphate- mucus test [61]	Bovine	5 ml saturated CuSO4 + 0.25 ml cervical mucus	Mucus turns rubbery and paste like
Sodium benzoate- urine test [55]	Bovine	3 ml of urine + 0.6 ml of Sodium benzoate	Green colour is reduced in 8-10 minutes
Fructose: Glucose ratio of mucus [62]	Bovine	Mass spectrometry Liquid and Gas Chromatography	Ratio of Fructose: Glucose should be 110: 20

5. Biological Tests

5.1 Aschiem Zondek test (A-Z Test):

This test identified the presence of pregnancy in mare and also to test hCG hormone in human female urine. For this test serum from test mare is injected 0.5 ml for 2-4 days subcutaneously or 5 ml intra peritoneally into 2-3 female rats of 22 days of age. Rats are killed at 96 to 120 hr after subcutaneous injection or 72 hr when injected intra peritoneally. Positive result is indicated by the presence of multiple corpora haemorrhagica on the ovaries and uterine edema.

Day of detection: 90 percent accurate when performed between the 60 to 100 days of pregnancy in the mare [63, 64].

5.2 Friedman rabbit test

2 ml serum from test mare is injected Intra Venous in female rabbits aged 14-20 weeks. Rabbits are housed separately and laparotomy is performed after 24 hours of injection.

Result: Positive result is indicated by the presence of multiple corpora haemorrhagica on the ovaries and uterine edema.

Day of detection: 90 percent accurate when performed between the 60 to 100 days of pregnancy in the mare [65].

5.3 Toad test

Toads like Bufovalliceps and frogs like Ranapipiens are used in this test. The principle of this test is that the sperm cells are emitted by toads/frogs only when stimulated with female frogs or gonadotrophins hormones.

Two male toads are taken for test and their cloaca is cleaned with normal saline and examined for presence of spermatozoa. If no sperms are present in the cloaca, 1 ml of test serum from a mare is injected into the dorsal lymph sac of the 2 male toads thrice at 1 hour interval. Then cloaca is examined for the presence of the sperms after 6 hours of the last injection. Positive for pregnancy is indicated by the presence of sperms in the cloacal region within 1 to 6 hours after the last injection [66].

Day of detection: 60 - 90 days of gestation [67]

5.4 Mouse test

The test is used to detect the estrogens secreted by the fetus, which is present in the urine or serum of pregnant mare.

Species: Equine [68]

Principle: The estrogens present in the urine or serum of pregnant mares produces estrous like changes in the reproductive tract of ovariectomized mouse.

Day of detection: 150 days of gestation

Accuracy: 70-80%

Procedure: 5-7 ml of serum or urine is collected from mare suspected to be pregnant. After collection, the urine is concentrated and detoxified. This is injected in ovariectomized mature mouse or rat subcutaneously on dorsal surface of neck. The test will be judged negative if no change is seen in the mouse/rat genitalia. A positive test for pregnancy would show the following changes in the mouse viz. vaginal edema and mucus discharge and the appearance of cornified or anucleated cells.

6. Imaging techniques

6.1 Ultrasonography

Ultrasound is considered to be the gold standard for determining pregnancy and confirming presence of viable fetus [1]. During recent year ultrasonography has become a choice for imaging the various body organs especially reproductive organ. It is less harmful than the radiography technique. Nowadays portable ultrasonographic machines are available for imaging the body for the presence of fetus. Ultrasound waves are inaudible to the human ear and operate at frequencies of 1 to 10 megahertz (MHz). Two types of ultrasound are employed in human and veterinary medicine: Using the Doppler phenomenon and the pulse-echo principle.

Basic principle: Ultrasound wave are generated by electrical pulse generator which strike the object and gets reflected. The reflected wave is captured by the receiver, which then gets converted into electrical signal and is visualised in the screen. The fluid filled structures appear black (anechoic), hard structures (like the bone) appear white (hyper echoic) and other structures with their structure midway between the bone and fluid appear grey (hypo echoic). The basic diagnosis of pregnancy lies in the identification of structures from black, grey or white scale.

Table 6: Ultrasonographic findings for early detection of pregnancy in different animal species

Species	Earliest day after mating	Technique	Placement of transducer	Diagnostic criteria
Horse [69]	9	B-mode RT	Transrectal	Embryonic vesicles
Cattle [70,71]	12	B-mode RT	Transrectal	Embryonic vesicles
	20	B-mode RT	Transrectal	Embryo, Heart beat
Buffalo [72]	30	B-mode RT	Transrectal	Embryo, Fetal fluids
Sheep and Goats [73]	60	Doppler A-mode	Transabdominal	Fetal Heart Sound, Fetal Fluid
	45-50	B-mode RT	Transabdominal	Fetal placentomes
	20-22		Transrectal	Fetal fluids
Pig [74]	60	Doppler	Transabdominal	Fetal Heart Sound
	60	A-mode	Transabdominal	Fetal Fluids
	22	B-mode RT	Transabdominal	Amniotic Fluids
Dog [75]	21	B-mode RT	Transabdominal	Embryonic vesicles

6.2 Radiography

This method of pregnancy diagnosis is based on the identification of fetal skeleton on an X-ray plate. The method poses radiation hazards to the animal, operator and the foetus.

Species: Ovine, Caprine, Swine, Canine, Feline

Species	Day of detection	Diagnostic criteria
Sheep and goats [76]	70-90 days	Fetal skeleton
Dog [77]	35-42 days	Fetal skeleton
Pigs [78]	42-49 days	Fetal skeleton
Cats [79]	17-30	Fetal skeleton

7. Future prospects

Blood proteomics profile (i.e., large scale study of protein functions, protein expression, protein-protein interactions) monitoring is very essential in animals to determine the sequential changes that occur from the day of estrus to successful conception. This determination leads to discovery of molecules, which will perhaps be novel and specific to the physiological stage of the animal. In order to qualify as a marker for pregnancy, the candidate molecule should be able to accurately determine the pregnancy status as early as possible with minimum false positives or false negatives. Additionally, the biological marker for pregnancy should be

(i) Specifically upregulated or downregulated during pregnancy
(ii) Least affected by non-animal factors like feed, environment, and drug interactions
(iii) Having the ability to reflect age as well as viability of the conceptus
(iv) Present in easily accessible body fluids like serum, milk, urine and vaginal discharge
(v) Expressed over a considerable period of time to give ample time for diagnosis
(vi) Revealing the result immediately

There is limited information available for the proteomics data in relation to pregnancy determination. Hence, further investigations are needed for arriving at definite pregnancy detection method. The blood proteomics data generated during pregnancy diagnosis can be further applied to study fetal viability and genetic disorders.

8. Conclusion

Accurate Pregnancy diagnosis is important for optimizing production among the animals. The techniques used for diagnosis vary as per the species of the animal and may also give false positive or negative results. It is essential to establish a standard technique for each species which can help in early pregnancy diagnosis. Proteomic studies can be established as a future tool for early diagnosis of pregnancy.

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