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Different methods of pregnancy diagnosis in Buffaloes

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Abstract
Accurate and timely pregnancy diagnosis in buffaloes is critical for effective reproductive management, improved fertility rates, and enhanced farm productivity. Various diagnostic methods have been developed and implemented, each with unique advantages and limitations in terms of accuracy, cost, ease of use, and stage of pregnancy detection. Traditional methods such as rectal palpation and observation of non-return to estrus remain widely used due to their simplicity and low cost. However, these techniques may lack precision or require skilled personnel. Advances in technology have introduced more reliable methods including ultrasonography, which allows early and accurate detection, and hormonal assays (e.g., progesterone, PAGs), which offer valuable biochemical insights into pregnancy status. Emerging techniques such as molecular diagnostics**,** immunoassays, and biosensors are also being explored for their potential to provide rapid, non-invasive, and on-field diagnosis. This review article highlights the principles, applications, benefits, and constraints of various pregnancy diagnosis methods in buffaloes, aiming to guide veterinarians and farmers in selecting the most appropriate approach based on their specific needs and resources.

Keywords: Buffalo, pregnancy diagnosis, reproduction, tests, early detection

Introduction
Buffaloes (*Bubalus bubalis*) play a vital role in the livestock industry, particularly in South Asia, due to their significant contribution to milk, meat, and draught power. Efficient reproductive management is essential to maintain optimal productivity in buffalo herds, and early and accurate pregnancy diagnosis is a cornerstone of this process. Timely detection of pregnancy allows for better decision-making regarding feeding, breeding, culling, and overall herd health management.

Unlike in cattle, reproductive efficiency in buffaloes is often compromised due to factors such as silent heat, poor estrus expression, and seasonal breeding behavior. These challenges make pregnancy diagnosis even more critical in buffaloes to minimize calving intervals and improve reproductive performance. Over the years, several diagnostic techniques have been developed, ranging from traditional methods like non-return to estrus observation and per rectal palpation, to more advanced technologies including ultrasonography**,** hormonal assays, and molecular diagnostic tools.

Each method has its own advantages and limitations in terms of sensitivity, specificity, cost-effectiveness, and the stage of pregnancy at which it can be accurately applied. This article aims to provide a comprehensive overview of the various approaches available for pregnancy diagnosis in buffaloes, evaluating their principles, applications, reliability, and practicality under field and farm conditions. Understanding these methods is essential for veterinarians, animal scientists, and farmers seeking to optimize reproductive efficiency and productivity in buffalo husbandry.

Different Methods of pregnancy Diagnosis in Buffaloes

A) Non-Return to Estrus (NRE) method
Non-Return to Estrus (NRE) method is one of the simplest and earliest approaches used to detect pregnancy in buffaloes under field conditions [1]. It is based on the principle that a pregnant animal will not return to estrus after successful mating or artificial insemination. After insemination, if a buffalo does not exhibit signs of estrus during the expected next cycle (usually 18-24 days post-breeding), it is assumed to be pregnant [1]. This is because in pregnant animals, progesterone levels remain elevated, preventing the onset of a new estrous cycle. Observe the buffalo daily for behavioral signs of estrus (e.g., mounting, restlessness, bellowing, vaginal discharge) after insemination. If no estrus signs are observed within 18-24

days, the animal is presumed pregnant. In case of doubt, continue observation during the second and third estrous cycles. It is cost-effective and non-invasive method. It requires no special equipment or training. Useful for smallholder farmers or in low-resource settings. Not a confirmatory test — false positives may occur in cases of silent heat, Early embryonic death, Prolonged luteal phase or anestrus, can delay rebreeding if the animal is not actually pregnant, Accuracy is limited, especially in buffaloes, which often show weak or silent estrus

B) Transrectal Method of Pregnancy Diagnosis in Buffaloes

The transrectal method is one of the most commonly used and reliable techniques for early pregnancy diagnosis in buffaloes. It includes both transrectal palpation and transrectal ultrasonography**,** each varying in accuracy and applicability depending on the stage of gestation and the skill of the operator.

1. Transrectal Palpation: Transrectal palpation involves the manual examination of the reproductive tract through the rectal wall to detect physical changes associated with pregnancy, such as uterine enlargement, fluid accumulation, and the presence of a fetus or fetal membranes. It can usually be performed from 35 to 45 days post-insemination in buffaloes [1]. The examiner inserts a gloved, lubricated hand into the rectum and palpates the uterus and ovaries. Key signs of pregnancy include asymmetry of uterine horns, fluctuation (fluid) in one horn, presence of a corpus luteum on the ovary Palpable fetal parts **or** fremitus in later stages. It is low-cost and widely accessible. It does not require specialized equipment. It can assess other reproductive conditions (e.g., ovarian cysts, uterine infections). It requires experience and training**.** Risk of injury to the rectal or reproductive tract, especially in young or small animals. May give false positives or negatives in early gestation or with reproductive abnormalities

2. Transrectal Ultrasonography (TRUS): Transrectal ultrasonography uses high-frequency sound waves to create real-time images of the reproductive tract, allowing visual confirmation of pregnancy and assessment of fetal viability. It **c**an accurately detect pregnancy as early as 25-30 days post-insemination [1]**.** A linear or convex ultrasound probe is inserted into the rectum and positioned over the uterus. The screen display**s** anechoic (black) fluid-filled uterine horns**,** embryo/fetus**,** and later heartbeat as pregnancy advances. It is highly accurate and can-do early detection. Can confirm fetal viability by detecting heartbeat**.** Non-invasive and safe for both animal and operator. Can detect twin pregnancies**,** fetal age**,** and abnormalities [2]. Requires expensive equipment and skilled personnel. Less accessible in remote or low-resource settings.

Major transrectal findings by month (approximate)

1. **Month 1 (days 1-30):** Early embryonic/oviductal phase: uterus essentially intra-pelvic; size may be normal or only slightly increased. On TRUS the embryonic vesicle may be visible from ~day 26-30 and cardiac activity may be detected from ~day 28-35. Palpation is generally insensitive in this month [3, 4].
2. **Month 2 (days 31-60):** Amniotic/early fetal phase: pregnant horn becomes slightly enlarged and slippery; uterine wall thins as the lumen expands. Amniotic (allantoic) vesicle and embryo/fetal heartbeat are routinely detected by TRUS from day ~30-35 onward. Transrectal palpation sensitivity increases markedly after day ~45. Corpus luteum often present on one ovary [3, 5].
3. **Month 3 (days 61-90):** Rapid uterine enlargement: pregnant horn moves cranially and out of pelvis into abdomen; uterine size appreciably increased on palpation; chorio-allantoic membranes can be "slipped" between fingers in many cases (membrane slip typically palpable from ~35-90 days). TRUS shows growing fetus and placentomes begin to be evident [5].
4. **Month 4 (days 91-120):** Uterus palpably large and intra-abdominal; fetal contours may be palpable by skilled examiners; placentomes palpable as discrete nodules on uterine wall; uterine horn thickening reduces as fetus occupies uterine lumen. TRUS shows clear fetal biometry and placentomes [5, 6].
5. **Month 5 (days 121-150):** Mid-pregnancy: uterus large, high in abdomen; placentomes more prominent on palpation; fetal limb/fetal movements may be felt by very experienced palpators (depends on operator and animal condition). CL may regress later but early in mid-pregnancy a CL can still be present [3, 5].
6. **Month 6 (days 151-180):** Uterus occupies much of abdominal cavity; placentomes easily palpable; fetal parts occasionally palpable (hindlimb, back) in large cows/buffaloes depending on body condition; transrectal palpation becomes more difficult as uterus displaces cranially — ultrasonography is preferred for detailed assessment [5, 7].
7. **Month 7 (days 181-210):** Late mid-pregnancy: uterine size large, palpation of specific fetal parts less reliable as rumen and abdominal contents intervene; placentomes remain palpable; transrectal Doppler/ultrasound can assess fetal viability and uterine blood flow [5, 8].
8. **Month 8 (days 211-240):** Uterus high in abdomen; placentomes still present but palpation of detailed fetal structures through rectum is limited; transrectal approach gives reduced direct information — transabdominal ultrasound may be more useful for late pregnancy evaluations [5, 9].
9. **Month 9 (days 241-270):** Nearing term foetus large, uterine contents fill much of abdomen; palpation of placentomes possible but often difficult; signs of advanced pregnancy (uterine size, abdominothoracic contour, mammary changes) become evident on clinical exam. TRUS or transabdominal scanning can monitor fetal heart and position [5, 6].
10. **Month 10 (days 271-birth) final month:** Uterus at maximum size, rectal palpation gives limited extra information beyond confirming advanced pregnancy; external clinical signs (mammary enlargement, pelvic relaxation) and transabdominal ultrasound are typically used to assess fetal presentation and readiness for parturition [3].

Advantages of Ultrasonography in Buffalo Pregnancy Diagnosis: Early and accurate detection (as early as 25-30 days). Non-invasive and safe**.** Fetal viability assessment.Fetal sexing (with expertise and appropriate timing)**.**Monitoring of reproductive disorders **(**e.g., mummification, pyometra, embryonic death).

Laboratory Tests for Diagnosis of Pregnancy in Buffaloes: Laboratory-based pregnancy diagnosis methods are important tools in veterinary reproductive management, especially when early and accurate detection is essential. These tests are primarily based on hormonal**,** biochemical, or protein markers that change in response to pregnancy. While they may not always replace physical or ultrasonographic methods, they are valuable for confirmation**,** monitoring, and research purposes**.**

1. Progesterone Assay: Progesterone assay is a widely used, practical tool for pregnancy diagnosis and reproductive monitoring in buffaloes: progesterone (P4) rises after ovulation and remains elevated during a normal pregnancy, so measuring P4 in plasma/serum or milk allows indirect detection of pregnancy from about 18-24 days after insemination (milk sampling is non-invasive and suitable for on-farm monitoring). Common laboratory methods are radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA); ELISA-based milk tests are popular because they are cheaper and easier to run than RIA and can be adapted to strip/kit formats for field use. Test performance depends on sampling timing and interpretation: single high P4 values can indicate pregnancy but also occur with a persistent corpus luteum or following embryonic loss, so sensitivity/specificity are improved when samples are taken on recommended post-insemination days (for buffaloes many studies indicate days ~20-24 as useful) or when serial samples are combined, and results are often best interpreted alongside transrectal ultrasound or pregnancy-associated glycoprotein (PAG) assays to reduce false positives/negatives. Practical limitations include variability between animals, effects of milk fat/composition on milk assays, and occasional kit cross-reactivity; nevertheless, when used correctly (appropriate sampling schedule, validated assay, and awareness of limitations) progesterone assays remain a reliable component of a herd pregnancy-monitoring program [3, 10, 11].

2. Pregnancy-Associated Glycoproteins (PAGs): Pregnancy-Associated Glycoproteins (PAGs) are placental proteins produced by binucleate trophoblastic cells and secreted into the maternal circulation soon after embryo implantation in buffaloes. These glycoproteins belong to the aspartic proteinase family and can be detected in maternal blood as early as 28-30 days post-insemination, making them valuable biomarkers for early pregnancy diagnosis. PAGs are measurable in plasma or serum using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), and their concentrations increase progressively with gestation, peaking during late pregnancy and declining after parturition due to placental detachment and clearance from the bloodstream. The persistence of PAGs postpartum (up to 80-100 days) can limit their usefulness for immediate rebreeding decisions. However, their high specificity to pregnancy and ability to indicate embryonic or fetal viability (a drop in PAG levels may precede embryonic loss) make PAG assays superior to progesterone-based tests in diagnostic reliability. PAG assays have been successfully adapted for buffaloes by using homologous or heterologous antisera from bovine PAGs, with high correlation between plasma PAG levels and fetal presence confirmed via ultrasonography [12, 13]. Therefore, PAG determination is now considered a sensitive, specific, and non-invasive biochemical marker for early pregnancy diagnosis and monitoring fetal well-being in buffaloes.

3. Estrone Sulfate Assay: Estrone sulfate (E_1S) assay is an important endocrine tool used for pregnancy diagnosis and monitoring fetal well-being in buffaloes. Estrone sulfate, a conjugated estrogen, is produced primarily by the feto-placental unit during gestation, with concentrations in maternal circulation and milk reflecting placental and fetal activity. In buffaloes, estrone sulfate becomes detectable in plasma or milk around day 100-120 of gestation, increasing progressively until term. Measurement of estrone sulfate is performed through radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), with RIA offering high sensitivity and specificity. The concentration of E_1S serves as an indicator of viable pregnancy—its sustained rise is associated with normal fetal development, whereas declining or low levels may indicate fetal death or placental dysfunction. Because of its later appearance in pregnancy compared with progesterone or pregnancy-associated glycoproteins (PAGs), the estrone sulfate assay is particularly useful for confirming mid-to-late pregnancy and monitoring fetal viability rather than for early detection. Furthermore, milk estrone sulfate levels provide a non-invasive and practical approach for field diagnosis in dairy buffaloes, showing strong correlation with plasma concentrations. Overall, the estrone sulfate assay is a reliable, hormone-based method to confirm pregnancy beyond the third month and assess fetal health in buffaloes [12, 14, 15].

4. Early Conception Factor (ECF) (Experimental): Early Conception Factor (ECF) is a pregnancy-specific immunosuppressive glycoprotein detected in the maternal circulation shortly after fertilization, making it one of the earliest known indicators of conception in buffaloes and other mammals [16]. ECF appears in the serum within 6-24 hours after fertilization and remains detectable for about 7-14 days post-conception in non-pregnant animals (if embryonic loss occurs) or throughout gestation in confirmed pregnancies. It functions by modulating the maternal immune response to prevent rejection of the developing embryo, thereby facilitating implantation and early embryonic survival. Detection of ECF is typically performed using the rosette inhibition test (RIT) or enzyme-linked immunosorbent assay (ELISA), both of which measure the suppression of lymphocyte reactivity induced by ECF. In buffaloes, ECF has been successfully detected as early as day 1-3 post-insemination, preceding measurable changes in progesterone or pregnancy-associated glycoproteins. Despite its potential as an ultra-early pregnancy biomarker, limitations such as the need for specialized assays, variability in immune responsiveness, and short diagnostic window have restricted its practical field application [12]. However, ECF remains a valuable research tool for early conception detection and embryonic survival studies in buffalo reproductive physiology. Diagnosis using this factor is still under research; not commercially standardized or widely used.

5. Relaxin Hormone Assay (Less common in buffaloes) Relaxin is a peptide hormone belonging to the insulin-like growth factor family, secreted primarily by the corpus luteum and placenta during pregnancy in buffaloes [12]. It plays an essential role in maintaining gestation, promoting uterine relaxation, cervical softening, and pelvic ligament loosening before parturition. In buffaloes, relaxin concentrations are low during early pregnancy, begin to rise markedly during mid to

late gestation (around 5-6 months), and peak near parturition, after which levels rapidly decline postpartum. Measurement of relaxin is usually performed through radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) using heterologous antisera derived from porcine or bovine relaxin, due to the high structural homology among species.

Relaxin's diagnostic potential in buffaloes lies mainly in confirmation of advanced pregnancy and prediction of parturition, rather than early gestation detection. Studies have shown that plasma relaxin levels correlate strongly with fetal number and placental mass, suggesting a role as an indicator of placental function and fetal well-being. However, its concentration remains below detection limits in non-pregnant or early pregnant buffaloes, limiting its practical use as an early pregnancy biomarker. Despite these constraints, relaxin assays provide valuable physiological insights into buffalo reproduction and have potential application in late-gestation monitoring and peripartum management.

Conclusion

Accurate and timely diagnosis of pregnancy in buffaloes is a crucial component of effective reproductive management, directly influencing herd fertility, productivity, and economic returns. A wide range of diagnostic methods—ranging from traditional approaches such as non-return to estrus observation and rectal palpation, to advanced techniques like transrectal ultrasonography and laboratory-based hormonal assays—are available to veterinarians and livestock managers. While non-return to estrus and rectal palpation remain accessible and practical under field conditions, methods like ultrasonography and pregnancy-associated glycoprotein (PAG) assays offer greater accuracy and allow for early detection and assessment of fetal viability. The choice of diagnostic method should be based on resource availability**,** stage of gestation**,** technical expertise**,** and the need for precision**.**

An integrated approach that combines multiple methods—particularly in high-value or intensively managed herds—can enhance the reliability of diagnosis and support better decision-making in reproductive planning. Ultimately, improving the efficiency of pregnancy diagnosis in buffaloes contributes significantly to optimizing reproductive performance and maximizing the profitability of buffalo farming systems.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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