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Abstract

The current study presents a pioneering scientific advancement that tackles the difficulties encountered by the agriculture sector in the timely identification of pregnancy in cattle. The primary goals of our novel urine-based pregnancy test are to minimize the occurrence of unproductive cow days, enhance productivity, optimize profitability, and provide precise and prompt outcomes. The examination relies on a unique interaction between urine and sodium hypochlorite, specifically activated inside an embryo or fetus. Our findings indicate that it is possible to identify pregnancy as early as 16 to 25 days after insemination. The sensitivity of this detection method is 97.39%, meaning that it accurately identifies pregnant cows. The specificity of the process is 73.33%, indicating that it correctly identifies non-pregnant cows. Overall, the method's accuracy is 93.33% for pregnant cows and 88% for non-pregnant cows. Our examination will be the initiation of a significant advancement, owing to its adaptability and cost-efficiency, which will render it a feasible option for breeders. This will result in reduced strain on cattle and a transformative approach to early pregnancy identification within the scientific domain.

Introduction

Pregnancy identification is crucial in enhancing cattle efficiency and boosting farmers' income. Early identification of non-pregnant cattle is of utmost importance, as it allows for prompt decision-making and appropriate management of the animals, such as re-breeding or culling. By adopting this proactive strategy, economic losses are minimized, and the cattle sector's overall

efficiency is enhanced worldwide. Early pregnancy evaluation is an essential technique in controlling fertility since it enables the prompt identification and isolation of pregnant animals from non-pregnant ones (Bahuguna et al., 2022). However, depending just on observing the absence of heat 21 days after mating could be more reliable. Therefore, it is necessary to utilize fast and reliable measures to avoid misdiagnosis. Various direct and indirect diagnostic techniques have already been devised to meet the need for precise early pregnancy detection in cattle (Gnemmi et al., 2022).

These techniques provide vital insights into a cow's reproductive condition, enabling farmers to make educated choices to enhance their breeding programs and overall farm administration. Regrettably, none of these techniques can detect a pregnancy before starting the subsequent oestrous cycle (Fodor et al., 2018).

The primary obstacle is promptly identifying non-gravid cows to enhance their monitoring or provide treatment to facilitate subsequent conception. Early identification of this phenomenon aids in minimizing the time between calving periods, thereby maximizing milk production throughout the cow's lifespan (Barbry et al., 2012). Practitioners and veterinarians often use several diagnostic techniques, including direct approaches like trans-rectal palpation and trans-rectal ultrasonography (TUS). However, accurately diagnosing pregnancy before day 30 of gestation using rectal palpation may be difficult (Dana et al., 2021).

The identification of early pregnancy in cattle continues to be a notable obstacle (Simon et al., 2008). Although there have been significant attempts to create diagnostic tools for early diagnosis of pregnancy in cattle, no effective approach has been found so far (Rawat et al., 2016). Alternatively, indirect diagnostic tools include methods like the Early Conception Factor (ECF) lateral flow test and Doppler color ultrasonography (Ambrose et al., 2007; Madog et al., 2022).

The accuracy of the ECF lateral flow test in diagnosing the non-pregnant state of dairy cows has been evaluated (Ambrose et al., 2007). Color Doppler ultrasonography is a novel technique that may detect non-pregnancy in animals at an earlier stage than other approaches, as shown by Gnemmi et al. (2022) and Madog et al. (2022). Although Radioimmunoassay (RIA) and Enzyme-Linked Immunosorbent Assay (ELISA) have been devised to identify chemicals like progesterone, Early Pregnancy Factor (EPF), and Pregnancy-Associated Glycoproteins (PAG), their precision does not achieve a 100% accuracy level (Bahuguna et al., 2022).

Several studies have used various biological samples, such as blood, milk, faeces, saliva, urine, and plasma, to ascertain the pregnant status of cows (Isobe et al., 2005; Samsonova et al., 2014; Dana et al., 2021). Previously, it was believed that urine did not include proteins. Later, it was shown that they exist in urine in minimal amounts and only rise under certain physiological or pathological circumstances (Bahuguna et al., 2022). Urine is regarded as an optimal source of biological material for identifying biomarkers due to its non-invasive nature compared to other

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bodily fluids (Adachi et al., 2006). Urine in cattle is a suitable medium for examining the diverse expression of urinary proteins under different physiological situations, such as pregnancy and various illnesses (Zheng et al., 2013; Rawat et al., 2016). Given these results, it would be intriguing to develop a diagnostic methodology that allows for prompt and dependable pregnancy identification (Commun et al., 2016).

This test is derived from the wisdom passed down by grandmothers. Since 1920, pregnancy tests have been accessible to women and evolved into a dependable means of obtaining information. Various tests have been conducted to determine the duration of pregnancy and reassure women, owing to the physiological similarities between cows and humans.

Presently, there is a lack of studies about the involvement of sodium hypochlorite in the identification of pregnancy in cows. Therefore, we implemented a testing procedure that used sodium hypochlorite in the urine of 145 dairy cows and compared the outcomes to those acquired by the reference technique, trans-rectal ultrasonography (TRU). This research aims to investigate the effects of sodium hypochlorite on cow pee during pregnancy and non-pregnancy, specifically focusing on detecting early pregnancy phases. It should be noted that this study is a first effort in this area. We undertook further study to analyze and describe the test findings, which included introducing sodium hypochlorite to cow pee to diagnose pregnancy. After conducting several experiments, these traits were consolidated into four criteria to simplify the analysis of test outcomes for operators. Remembering that any new test will go through several phases of improvement before reaching its ultimate edition is crucial. This novel examination offers a helpful supplement, especially advantageous for emerging nations.

In light of this context, this research aimed to create and evaluate a cost-efficient, moderately precise, and relatively fast urine-based pregnancy test.

Materials and methods

Materials

Portable ultrasound machine, urinary probe, sponge, antiseptic 5% Chlorhexidine, basin, warm water, clean empty, glove for transrectal palpation, cube for sampling, 10cc syringe, and sodium hypochlorite 16% (Figure 1).

Figure 1. Materials used for gestation diagnostics

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Animal selection

We conducted a study on a sample of 145 dairy cows chosen randomly and in good health. The selection criteria included primiparous and multiparous cows from the Prim' Holstein and Blanc Bleu Belge breeds, ages 2 to 9 years. The cows were obtained from several educational dairy farms in Belgium, namely "The educational farm of the Liège Veterinary Institute in Belgium" and "The Liège pilot farm specializing in milk production," as well as two pilot farms in Constantine, Algeria (Table 1).

Table 1: Criteria for identifying animals used in experiments: Animal types and study designs

Selection Criteria	Categories	Experimental Protocol
Biological	Sex	Female
	Age	Adults & heifers
	Breed	Belgian Blue &Prim'Holstein
Feeding	Feeding	Balanced
Clinical	Clinical history	Absence
Geographic	Location	Educational farm of the Liège, Veterinary Institute, Belgium

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Throughout the cow selection procedure, we eliminated animals who had shown clinical symptoms of periparturient illnesses or were determined to be in poor condition according to a veterinary clinical assessment. In addition, we considered the physiological stage of each animal. None of the chosen cows had any discernible abnormalities in their reproductive systems, which was established by analyzing records and doing transrectal palpation.

Urine sampling and processing

The research adhered to the ethical criteria set by the Institutional Animal Ethics Committee of the Veterinary Institute of Liege, Belgium, regarding animal care and sample handling methods. The experimental technique used for this investigation adheres to the guidelines set out by the ASVCP (Friedrichs et al., 2012).

Following artificial insemination (AI), urine samples were collected at various intervals and stages of gestation, ranging from the 10th day to the 9th month of pregnancy. Specimens were collected by unforced micturition. Urinary catheterization was carried out when spontaneous or induced micturition was impossible. The pre-analytical elements of our investigation are listed in Table 2.

Table 2: Pre-analytical aspects within research: Relevant protocols and criteria concerning the handling of urinary samples

Pre-analytical Factors	Categories	ExperimentalProtocol
Collection period	Time of year	October-November 2019
	Time in the day	Morning at 8 AM
Animal preparation	Restraint	Assisted by the breeder
	Cleaning	Clearwater and thorough wiping of the vulva
Equipment preparation	Disinfection	5% Chlorhexidine (contact time > 1 hour)
	Materials	Urinary test strips FEPEX

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The Abbott Alinity ci-series system analyzer

Sample collection	Methods	Urinary catheterization or spontaneous micturition
	Manipulator	Pr. Christian Hansen
Sample handling	Transport	< 2 hours between collection and CHU
	Temperature	4°C in a cooler
	Centrifugation	
		EBA 3S centrifuge; 1900 RPM for 5 minutes
Specimen stability	Storage	4°C in a fridge and/or cooler

95% CI, 95% confidence interval.

a In the 'extra wash' method, six individual washes (vs. three washes recommended by the manufacturer) were used for each of the two wash steps.

Test technique

Urine collection after vulva cleansing with water. Each animal contributed a urine sample ranging in volume from 8 ml to 20 ml, which was meticulously collected using a sterile 20 ml syringe. To maintain the authenticity of these samples, they were promptly gathered in a sample tube and combined with a solution consisting of two-thirds cow urine and one-third sodium hypochlorite solution (16%). After a little interval, the outcome is recorded. A positive test is confirmed by observing vigorous bubbling and forming a white ring with a powdery consistency (Figure 2).

Figure 2. Example reaction for test sodium hypochlorite with urine and formation the chalky texture (Positive reaction).

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Pregnancy confirmation

Following the urine sodium hypochlorite test, pregnancy was established using trans-rectal palpation (Figure 3) and further verified using trans-rectal ultrasonography in the days after reproduction. As a result, the animals were categorized into two groups: pregnant cows and non-pregnant cows.

Figure 3: Transrectal palpation (Christian hanzen).

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The choice of a pregnancy diagnostic technique is generally determined by three fundamental factors: prompt identification, convenience, and precision. The evaluation of pregnancy diagnostic techniques includes examining four factors: sensitivity, specificity, and the accuracy of both pregnancy and non-pregnancy diagnoses. The first two criteria are used to assess the approach itself, whilst the latter were applied to evaluate the competence of individual users (Hanzen, 2015).

Test results are categorized into one of the following groups:

- True positive (TP): positive test and pregnant cow on ultrasound.

- True negative (VN): negative test and non-pregnant cow at ultrasound scan.

- False positive (FP): test positive but cow not pregnant on ultrasound.

- False negative (FN): test negative but cow pregnant on ultrasound.

From this labeling, the sensitivity (Se) and specificity (Sp) of the test, as well as the gestational and non-gestational accuracy, were derived. The sensitivity of each test was measured as 100× (TP/VP+FN), while the specificity of individual assays was determined as 100× (TN/TN+FP). The accuracy was calculated as 100× (TP+TN/TP+TN+FP+FN). Accuracy for pregnant cows was derived as 100× (TP/TP+FP), whilst accuracy for non-pregnant subjects was determined as 100× (TN/TN+FN) (Dana et al., 2020).

Results and Discussion

A search for signs of pregnancy in 145 cows was conducted utilizing direct and indirect pregnancy tests. The information is shown in Table 3. Please provide a detailed analysis of the findings, categorizing them as true positive, true negative, false positive, and false negative, for the evaluation of pregnancy using the sodium hypochlorite test, in contrast to the gross inspection of the genital canal as the standard.

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The results of the sodium hypochlorite test indicated that 112 out of the 145 cows (77.24%) were accurately identified as pregnant, whereas 22 (15.17%) were identified as non-pregnant. The sodium hypochlorite test yielded eight erroneous positive diagnoses (6.9%) and three false negative diagnoses (2.6%). The data may be seen in Table 3.

The test's accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are shown in Table 4. The sensitivity of the sodium hypochlorite test for identifying pregnancy was 97.39%, while its specificity was 73.33%. The test has an accuracy rate of 92.41% in detecting pregnancy. The test had positive and negative predictive values of 93.33% and 88%, respectively. In our sample, the likelihood of a cow being pregnant is 93.33%, while the probability of a cow not being pregnant is 88%.

The objective of this research was to ascertain and conduct an initial assessment of the impact of sodium hypochlorite in diagnosing pregnancy in cows using urine analysis. The findings demonstrated that our urine pregnancy test exhibited outstanding accuracy, with a sensitivity rate of 97.39% and a specificity rate of 73.33%. The test showed a noteworthy precision of 93.33% for pregnant cows and 88% for non-pregnant animals.

The evolving cattle sector may impact the future methods of doing pregnancy diagnosis. The increased focus on reproductive management in beef herds and artificial insemination (AI) leads to a greater need for precise and rapid pregnancy diagnosis (Lucy et al., 2011). Identifying non-pregnant cows is essential for reducing the interval between calving and initiating a future pregnancy, enhancing milk production, and increasing farmer profitability. Various diagnostic techniques are readily accessible and often used in agricultural settings. Ultrasonography and other traditional methods, such as rectal palpation, are often used for the diagnosis of pregnancy in cattle. The precision of rectal palpation may be inadequate before 30-35 days of pregnancy, and there is a potential danger of inducing abortion during its use (Janjanam et al., 2014). The use of trans-rectal ultrasonography for pregnancy detection is common, although it requires the skills of specialist professionals (Garcia et al., 1993). The use of ELISA and radioimmunoassay methods for pregnancy detection using blood, milk, and feck samples has been extensively studied. These techniques need the use of radioisotopes and the collaboration of suitable labs to interpret the findings. The sodium hypochlorite test does not need these characteristics for diagnosing pregnancy since it allows for the simultaneous evaluation of several samples in a short time frame, even under field settings.

The cattle sector is increasingly adopting alternate techniques for pregnancy detection that do not need the expertise of professionals or specific equipment. The sodium hypochlorite test provides this capability; this technique may be readily implemented in the field and does not need a dedicated laboratory. This technique involves the simple combination of a tiny quantity of urine with sodium hypochlorite, allowing for easy testing of samples. The outcome becomes visible within a matter of minutes.

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Pregnancy may be detected by measuring several molecules in the blood at different periods after insemination. On the second day, the ECF test was initially deemed reliable, but further analysis revealed that the test may be defective at any moment. Other examinations evaluate the physiological reaction to progesterone in blood or milk during days 18 to 23, interferon- during days 16 to 18, or pregnancy-related glycoproteins (PAG) after day 25 (Lucy et al., 2011). The sensitivity of the Sodium hypochlorite test for pregnancy detection in this study was 97.39%. The result aligns with the findings of (Akkose et al., 2023), who reported a 97.4% accuracy rate for the IDEXX rapid visual pregnancy test (RVPT).

On the other hand, different scientists observed that the Bovine Pregnancy Rapid Test (UbioquickVET; BPRT) succeeded 89.4% in diagnosing early pregnancy in dairy cattle. This was determined by detecting the presence of the PAG (pregnancy-associated glycoprotein) between 30 and 40 days after artificial insemination. Another study conducted by Moussafir et al. in 2018 and Dana et al. in 2020 found that the sensitivity of the barium chloride test was 79.4%. These rates were lower than the results obtained in the present study.

Nevertheless, the outcome is inferior to the one obtained from the commercial ELISA-PAG test (Bovine Pregnancy Test DG29®), which demonstrated a sensitivity of 100%, according to Moussafir et al. (2018) and Dana et al. (2020). The P4 ELISA test yielded identical results to those reported by Moussafir et al. (2018) and Dana et al. (2020). The current study shows that the Sodium hypochlorite test's specificity was 73.33%. The obtained result is similar to the progesterone ELISA finding reported by Dana et al. (2020) for detecting non-pregnant cattle, which was 62.5%. Nevertheless, the authors also noted a reduced specificity of 30.0% for the barium chloride test. Furthermore, our results demonstrate a decreased performance compared to the findings reported by Moussafir et al. (2018) for the tests BPRT and DG29 in diagnosing non-pregnant animals, with success rates of 89.8% and 81.3%, respectively. The Akkose et al. (2023) study reported comparable outcomes for the Rapid Visual Pregnancy Test and On-Farm Pregnancy Test, with accuracy rates of 92.1% and 93.1%, respectively. The Sodium Hypochlorite Test in our experiment yielded a Positive Predictive Value (PPV) of 93.33%. This indicates that the accuracy of our test in identifying pregnant cows is higher than the 87.4% and 89.1% accuracy achieved by the Rapid Visual Pregnancy Test and On-Farm Pregnancy Test, respectively, as reported by Akkose et al. in 2023. However, the test's accuracy for non-pregnant cows (NPV) was lower at 81.2% compared to the 94.0% and 95.6% achieved by the same author in their previous studies. In this investigation, the Sodium Hypochlorite Test demonstrated an accuracy of 92.41%, surpassing the reported accuracies of 79.7% by Dana et al. (2020), 84.8% by Ghaidan et al. (2019), and 87.0% by Silva et al. (2007) when employing progesterone ELISA. The barium chloride test had a lower rate of 52%, as reported by (Dana et al., 2020), which is lower than the rate discovered in our research. Nevertheless, the accuracy value shown in the present investigation is consistent with the accuracy value (94%) published by Moussafir et al. (2018) for the PAG-ELISA test conducted 30-40 days after artificial

insemination (AI). In their study, Ricci et al. (2015) found that using PAG-ELISA 27 days after artificial insemination resulted in a similar outcome, with a success rate ranging from 94% to 96%. Administering the same examination 32 days after artificial intelligence. Humblot et al. (1988) demonstrated a 92% accuracy rate in their findings using the PAG-ELISA test.

The disparity between the accuracy of the present research and those published in the literature may be attributed to the variation in the timing of sample collection after artificial insemination. It is known that accuracy tends to increase as pregnancy progresses.

The significance of promptly identifying pregnancy in the early stages is of utmost relevance in cow farming. Through accurate identification of gravid cows during the early stages, farmers may enhance their livestock care and operational strategies, leading to a reduction in the number of unproductive cow days and a boost in overall output. Moreover, timely identification of non-gravid cows enables farmers to make well-informed choices on breeding, enhancing the farm's overall profitability. The test's ease and exceptional accuracy rates make it an appealing and economically efficient option for farmers, especially those engaged in large-scale operations. Additional studies are necessary to finalize the research and ensure its economic viability and reliability throughout this first round of the test.

Conclusion

The addition of the sodium hypochlorite (16%) examination in the current investigation provides a dependable, practical, straightforward, cost-effective, and notably prompt substitute for urine-based pregnancy detection. The detection of early pregnancy in cows at day +16 after AI is both non-invasive and dependable up to D16. This methodology represents a significant breakthrough in diagnosing cow pregnancies, but confirmation by an extra test is still necessary.

Nevertheless, this investigation will be extended via further examinations of the interaction between sodium hypochlorite molecules and urine from gravid bovines, advancing the progression toward a comprehensive commercial study and the expeditious development of a commercially accessible test.

Conflict of interest statement

Acknowledgment

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the herd veterinarians and extend special appreciation to Professor Christian Hanzen for
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Belgium.

Data Availability Statement

Data are available upon reasonable request.

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