Journal of Advanced Zoology
ISSN: 0253-7214
Volume 45 Issue 6 Year 2024 Page 69-78

# Evaluation of Physical Properties of Saliva as Non-Invasive and Point-Of-Care Diagnostic Tools for Early Pregnancy Detection in Cows

**Farzana[1], Ali Gohar[2], Maaz Ahmad[1], Raheela Taj[3], Muhammad Hanif[1], Niamat Ullah[4], Mansoor Ahmad[1], Zulfiqar Ali[1], Aziz Ullah Khan[1], Asad Ullah[2], Imad Khan[2]***

*[1,3,5,7,8,9,]Department of Zoology, Abdul Wali Khan University Mardan, 23200 Pakistan.*
*[2,10,11*]College of veterinary Sciences and Animal Husbandry Abdul Wali Khan University Mardan, 23200 Pakistan.*
*[4]Institute of Chemical Sciences (ICS), University of Peshawar 25120, KP Pakistan.*
*[6,]Civil Veterinary Hospital Loralai, Balochistan, 84800, Pakistan*

***Correspondence:** Imad Khan[2]*
**imadkhan@awkum.edu.pk*

***Abstract***

The study aimed to create a cost-effective pregnancy detection model for cattle by combining early pregnancy predicting parameters and focusing on changes in saliva's physical properties. Saliva from 100 pregnant and 100 non-pregnant cows in Charsadda, Khyber Pakhtunkhwa Pakistan, were collected using pre-weighed sponges attached to a thin, flexible metal rod placed in the cows' mouths for 30 seconds, then processed at farms. Results showed that pregnant cows had significantly higher mean pH (9.325 ± 0.13) than non-pregnant cows (8.133 ± 0.13). Conversely, non-pregnant cows exhibited higher mean specific gravity (0.000173±0.00) and conductivity (0.666 ± 0.029) compared to pregnant cows (0.000146±0.00 and 0.538 ± 0.028). Additionally, non-pregnant cows had higher buffer capacity (7.40 ± 0.10) and flow rate (91.92±1.13) than pregnant cows (2.42 ± 0.151 and 91.92±1.13). Six distinct salivary crystallisation patterns were identified: branches, ferns, fir, dots, none, and combinations. Fern-like (26.19%) and branch-fern (19.04%) patterns were predominant in pregnant cows, while branch-fir (29.03%) and branch-fern (22.58%) were common in non-pregnant cows. These variations are likely influenced by hormonal changes during pregnancy. Further research is needed to validate these findings for early pregnancy diagnosis in cattle and potentially other animal species.

***Keywords:*** *Biomarker, early pregnancy, non-invasive, physical properties, saliva.*



## 1. Introduction

Reproductive efficiency is a key element in livestock production. Effective management of breeding in livestock enterprises is directly related to enhanced productivity and overall profitability of cattle operations. Diagnosis of pregnancy at an early stage is critical for effective management of breeding cycles. It allows for timely treatment of open animals and rebreeding to maintain a post-partum barren interval of 60 days (Balhara et al., 2013; Skalova et al., 2013). This results in an increase in the number of calves born each year, ultimately boosting the productivity of the herd. On the other hand, failure to diagnose non-pregnant animals at the earliest

will led to extended days open period resulting in declined production output. Livestock farming requires strategic allocation of resources such as feed, labor and infrastructure to improve the health and well-being of cattle. Early pregnancy diagnosis is the utmost important for optimal resource allocation. A pregnant cow can be allocated to an appropriate nutritional group to meet her nutrients demand for gestation. In contrast, open cows can be managed differently to reduce the operational cost. Early pregnancy diagnosis can provide valuable information about repeat breeder and non-productive animals in the herd. These animals can be culled to maintain the overall reproductive health of the herd. The establishment of pregnancy initiates an interaction between the conceptus and the maternal system, inducing controlled physiological, immunological, and hormonal changes that are essential for the sustainable growth and development of the fetus (Warning et al., 2011;Pillay et al., 2016).These changes also extend beyond the reproductive system and are reflected in the physical and compositional nature of bio-fluids, such as blood, saliva, tear, urine, sweat, and cerebrospinal fluids, providing a unique opportunity to explore potential biomarkers related to the pathophysiology of pregnancy (Barak et al., 2003).Among the various body fluids, saliva is considered a "mirror of the body" and a crucial source of indicators for local, systemic, and infectious phenomena(Yoshizawa et al., 2013).Therefore, there is a growing interest in exploring, identifying, and validating salivary biomarkers for clinical-diagnostic purposes, which has led to the foundation of an independent and emerging field of salivary diagnostics.

Several traditional methods such as rectal palpation and ultrasonography are currently used for pregnancy diagnosis in cattle. However, these methods are invasive, require skilled personnel, and can be costly. Thus, there is a growing interest in developing non-invasive, cost-effective, and reliable methods for the detection of early pregnancy diagnosis in cattle. The non-invasive, easy-to-collect, store, and ship nature of saliva, along with its effectiveness as a diagnostic fluid, makes it a popular choice for pathophysiologic research (David and Wong, 2006). Numerous research studies have evaluated salivary biomarkers for the assessment of reproductive processes in various species. For instance, salivary progesterone concentration has been used to diagnose early pregnancy in sows (Mariyoshi et al., 1996) while variations in salivary secretion and physical properties of saliva have been correlated with circulating steroid hormone concentration during different stages of the menstrual cycle in humans (Ravinder et al., 2016; Devi et al., 2016). Saliva crystallization, also known as Ferning, in response to reproductive hormones, has shown potential for early pregnancy diagnosis in cattle (Skalova et al., 2013). Furthermore, pregnancy-induced changes in salivary biochemistry, including changes in the concentration of calcium, phosphorous, and total protein (Bakhshi et al., 2011).

Saliva is a promising bio-fluid for early pregnancy diagnosis in cattle due to its non-invasive nature, easy-to-collect, and storeable nature. Its effectiveness in diagnosing pregnancy makes it suitable for point-of-care testing systems, enabling on-site diagnosis in remote areas with limited veterinary services. This could improve reproductive efficiency and increase profitability. Therefore, more research and investment in this area can have significant benefits for livestock management and the agricultural industry.

This research aims to investigate the potential of saliva as a valuable bio-fluid for studying the pathophysiologic aspects of pregnancy and reproductive processes in cattle. The focus is on understanding the changes in physical properties of saliva during early pregnancy. The primary objective is to compare these changes and subsequently develop a cost-effective point-of-care testing system for early pregnancy diagnosis in cattle. This system is intended to be user-friendly for both veterinary clinicians and farmers, providing a practical tool for timely and efficient pregnancy detection in cattle.

## 2. Material and Methods

### 2.1. Study Design

A cross-sectional investigation design was employed consisting of saliva samples collection from 100 pluriparous Holstein Friesian pregnant cows within 25-35 days of gestation range and an equivalent number of 100 non pregnant cows. Selection criteria ensured that all the cows were free from diseases and were maintained under identical management conditions. The average body weight of cows included in the study was 600-800 kg. The study encompassed various farms for samples collection in district Charsadda, including Govt.

Cattle Breeding and Dairy Farm Harichand as well as private farms like Sucha Dairy Farm and Jan Dairy Farm, district Charsadda. The cows on all these farms were maintained under uniform management conditions. They received a standardized diet consisting of 70% wheat straw and silage, and 30% commercial Wanda (22% crude protein) of total dry matter intake. The research focused on evaluating several physical properties of saliva including pH, flow rate, crystallization pattern, buffering capacity, specific gravity, density and electrical conductivity.

### 2.2. Study Area

The current study was performed in different Dairy Farms of district Charsadda, Khyber Pakhtunkhwa, Pakistan. The laboratory activity was then performed on the collected saliva samples of the cow in Physiology laboratory at College of Veterinary Sciences and Animal Husbandry, Abdul Wali Khan University Mardan Pakistan.

### 2.3. Sample collection and investigation of parameters

The research focused on evaluating several physical properties of saliva including pH, flow rate, crystallization pattern, buffering capacity, specific gravity, density and electrical conductivity. Saliva samples were collected in the early morning hours before the cows received their morning feed. Saliva samples were collected in the early morning before the cows received their morning feed. The procedure for collecting saliva from the cow involved inserting into the cow's mouth pre-weighed sponges that were attached to a thin, flexible metal rod, (Contreras-Aguilar, 2021) for 30 seconds. The saliva laden sponges were weighed and subsequently placed into the barrel of a 20 ml disposable syringe and pressed with a plunger to squeeze the saliva into Eppendorf or a calibrated test tube. The debris and food particles trapped in the sponge and thus clear saliva samples were obtained. The pH of the samples was measured immediately after collection of saliva. After obtaining the saliva samples, the study parameters were performed as follows:

### 2.4. Detection of parameters of saliva

#### 2.4.1. Measuring pH Of Saliva

pH paper was used to measure the pH of the saliva of both pregnant and non-pregnant cows. The pH strip was dipped in each saliva sample. After waiting for 10 seconds, the strip was removed and shaken off to remove excess saliva. A color chart supplied by the manufacturer was used to compare the strip's color. MColorpHast (Germany) and the pH value was recorded for each sample.

#### 2.4.2. Salivary crystallization or ferning test

1. On a glass slide, a drop of saliva from each sample was applied, and it was allowed to air dry at room temperature. The slides were examined under a microscope at magnification of 100× objective lens (Labomed® LX made in USA) before being captured on camera. Six different distinct crystallization patterns were classified as none, branch-like, fern-like, fir-like, dotted, and typical fern-like (Skalova et al., 2013). Utilizing a portable camera pressed up against the compound microscope's ocular, the images were captured. The frequency of every pattern was noted and represented as a percentage of all the patterns. None of the branch-like and fern like crystallization were predominate in pregnant whereas in non-pregnant animals, mixed branch-like and fir-like or mixed branching, fir- and fern-like properties were seen.

#### 2.4.3. Buffering Capacity of Saliva

The buffering capacity of saliva in pregnant and non-pregnant cows in early pregnancy was measured by the Ericsson method as follows,

1. 2 ml of saliva sample was diluted with 2 ml of double glass distilled water.
2. The pH of the diluted saliva was then measured using a pH meter.
3. A buffer solution (0.05N HCl and 0.05N NaOH) with a known pH value was added to the diluted saliva in small increments. The pH of the mixture was measured after each addition of buffer solution. The quantity of buffer solution needed to raise the mixture's pH by one pH unit was used to calculate the saliva's buffering capacity.
4. The buffering capacity was calculated using the Henderson-Hasselbalch equation (Kivela et al., 2003) considered the initial pH of the diluted saliva, the pKa of the buffer solution, and the amount of buffer solution required to change the pH of the mixture by one unit. The summarized form of the Henderson-Hasselbalch equation is as follows.

$pH = pKa + \log([A^-]/[HA])$

$\Delta Acid$ = volume of HCl x molarity of HCl

$\Delta Base$ = volume of NaOH x molarity of NaOH

Buffing capacity = $(\Delta Base/\Delta Acid) \times (V/2)$

V= the volume of the fresh saliva sample

pKa = acid dissociation constant

### 2.4.4. Density of saliva

The density of equal volume of saliva of non-pregnant and pregnant cows were compared. For this purpose, the mass of 5 ml of saliva was measured, and the density of the saliva was determined using the standard formula. D = m / v (Brown et al., 1969).
Where **m** is the mass of saliva, **v** is the volume of saliva and **D** is the density of saliva.

### 2.4.5. Specific Gravity

It was determined what the specific gravity of the saliva was from pregnant and non-pregnant cows from the density of saliva using the following formula: Specific gravity (preg/non-preg) = Density of saliva / Density of water. So, the specific gravity of the saliva was determined by dividing the density of the saliva by the density of water (which is 1 g/cm³) and the results were compared between the pregnant and non-pregnant animals.

### 2.4.6. Saliva Flow Rate

Swabs that had been previously weighed and attached to a thin, flexible metal rod were inserted into each cow's mouth for 30 seconds to measure the saliva flow rate. The following methodology is outlined by. The weight of saliva produced was calculated by subtracting the weight of the pre-weighed unloaded (dry) sponge from the weight of the loaded (wet) sponge. The salivary flow rate was expressed as g/min.

### 2.4.7. Electrical Conductivity

The electrical conductivity of saliva from pregnant and non-pregnant cattle was measured and compared. An electrical conductivity meter was used for this purpose. The conductivity meter was calibrated according to the manufacturer's instructions HANNA instruments® Inc, (made in romania), using a standard solution of known conductivity distilled water to ensure accurate and consistent reading. The sample of saliva was diluted in distilled water (1 ml of saliva with 9 ml of distilled water) to obtain a suitable conductivity range for the meter. A disposable plastic cuvette was filled up to 2/3 of its capacity and inserted into the conductivity meter and waited for the reading to stabilize. The meter displayed the conductivity value in Siemens per meter.

### 2.5. Statistical Analysis

Data were analyzed statistically using appropriate social science statistical software (SPSS and t-test to ascertain the experiment's significance. The data was entered into Excel for statistical analysis to obtain the P-value. When P value $< 0.05$ the results were considered significant.

## 3. Results and Discussion

The present study was carried out to investigate the feasibility of utilizing saliva for a non-invasive and Point-of- Care diagnosis of early pregnancy in cattle. Saliva samples were collected from pluriparous HF cows (open and 25-35 days post inseminated) and subjected to biophysical analysis including comparison of pH, flow rate, buffering capacity, density, specific gravity, electrical conductivity and pattern of crystallization shown in Table 3.1. The results are presented as mean values and standard error, providing insights into the variations in these parameters between the two groups (Table3.1). The study outcomes can be summarized as follows: Comparing pregnant animals (PA) to non-pregnant animals (NPA), the pH values in the saliva of PA group are considerably higher ($p < 0.05$). However, there is no significant difference in the density of saliva between NPA and PA groups. The electrical conductivity measurements of diluted and undiluted saliva between NPA and PA were compared. A significantly higher ($p \leq 0.05$) electrical conductance was observed in both diluted ( $0.666 \pm 0.029$ ) and undiluted ($3.007 \pm 0.19$) saliva of NPA as compared to PA diluted ($0.5385 \pm 0.028$) and undiluted saliva ($2.42 \pm 0.151$) samples. Buffering capacity and flow rate exhibited no significant variation between NPA and PA, although, numerically the flow rate of saliva was higher in PA as compared to NPA. The specific gravity of saliva from NPA was significantly higher ($p \leq 0.05$) than PA. The saliva samples from NPA and PA were smeared and microscopically observed for different patterns of crystallization. Six different patterns of crystallization including none, doted, fern like, fir like, branch like and combination of two or more patterns were observed**.** The PA predominantly exhibited fern-like patterns (26.19 %) followed by a branch +fern (19.04 %) crystallization pattern. On the other hand, the predominant crystallization patterns in NPA were branch +fir (29.03%), branch +fern (22.58 %), branch +fern+ fir (19.35 %) and dot like (19.35 %).

### 3.1. Saliva Crystallization Different Pattern

The study in which examined the cows' salivary crystallization patterns during the pregnancy. Six different patterns were identified, including branches, ferns, fir dot none and a combination of these patterns was shown in Figure 3.1. In the pregnant group most of the ferns, branch-like pattern was seen. Moreover, non-pregnant group none, fir, dot and combination were seen. In pregnant cows, Fern-like patterns are 26.19, fir-like 4.76, and Branch-like 11.90, Dot-like 9.52, Branche-fir 7.14, Branche-fir-fern 4.76, branch-fern 19.04, fern-fir 7.14, none 9.52. In non-pregnant cows, fern-like 0.00, fir-like 6.45, bran-like 0.00, Dot-like 19.35, branch-fir 29.03, branch-fern-fir 19.35, branch-fern 22.58,fern-fir 0.00, none 3.22. All cows exhibited a fern-like pattern on the first measurement day, and pregnant cows had branch and fern-like patterns were observed. The estrogen and progesterone ratio positively correlated with fern-like patterns during pregnancy.

This research study was carried out to explore the potential biomarkers of early pregnancy detection in cattle through an examination of physical properties of saliva. By comparing the physical aspects of saliva (pH, buffering capacity, flow rate, density, specific gravity and electrical conductivity) in early gestating and non-pregnant cows, we have identified notable numerical and statistical variations. The observed variations suggest a biological response to early pregnancy in bovine species. These variations hold promise for the development of a point-of-care diagnostic tool tailored for early pregnancy detection in bovine species. Furthermore, the findings of our study can be used potentially for advancing the reproductive management practices in cattle.

The results of our study revealed a significant increase ($p < 0.001$) in pH and a non-significant but still numerically low buffering capacity in the saliva of pregnant animals compared to their non-pregnant counterparts. The phenomenon can be explained according to the fundamental principles of chemistry which states that a lower buffering capacity of a solution signifies its reduced ability to resist change in pH. In the context of our study, the diminished buffering capacity in the saliva of pregnant cows aligns with the higher pH noted. Furthermore, the explanation for higher pH and alkaline shift in the saliva of pregnant animals can be extended based on findings reported by Alemrajabi et al., (2021). They have reported a decrease in the levels of urea and glucose in the serum and saliva of pregnant women as compared to their non-pregnant counterparts. Urea is a neutral substance and hence has no effect on the pH of saliva. However, the metabolic end products of glucose are $CO_2$ and $H_2O$. The $CO_2$ get dissolved in water leading to the formation of carbonic acid ($H_2CO_3$). The low level of glucose in the saliva of pregnant women will result in the lower production $H_2CO_3$, leading to alkaline shift. Furthermore, it is possible that any amount of $H_2CO_3$ formed will dissociate in $H^+$ and $HCO_3$- ions. HCO3- is alkaline and may cause a rise in the pH of saliva in pregnant animals. The $H^+$ ion formed may drop down the pH if remained in ionized form. According to the Mekonnin et al., (2017), during the physiological di-estrus of pregnancy, the concentration of progesterone was found to be significantly higher ($p< 0.05$) in saliva than any other stage of reproductive cyclicity. According to National Library of Medicine (NIH), an official website of United States government, the hydrogen acceptance count of progesterone is 2 (HAC=2) at electronegative N-terminus. The electronegative terminus may accept the free $H^+$ and thus lower the $H^+$ concentration and neutralize its acidifying potential. The low concentration of $H^+$ concentration in the first and third trimesters of pregnancy has also been reported by Yousefi et al., (2020).

However, several researchers have reported a decreasing trend of pH during pregnancy (Karnik et al., 2015; Laine et al., 2000; Rockenbach et al., 2006; Saluja et al., 2014; Migliario et al., 2021). Considering the results of our study and findings by other researchers, one can say that the actual pH regulation in saliva involve multiple factors beyond the described interactions and it is influenced by the dynamic interplay of various substances present in saliva and surrounding environment.

In our study, although non-significant but numerically low saliva flow rate was observed in pregnant as compared to non-pregnant cattle. However, the impact of pregnancy on saliva flow rate is diverse and complex, marked by vast variability and lack of uniformity in the findings of researchers. A lower flow rate of saliva in human pregnancy was also observed by Rockenbach et al., (2006). Hedge et al., (2016) reported a significant decrease in salivary flow rate and pH during pregnancy compared to non-pregnant women. Similarly, Al–Nuaimy et al., (2003) reported decreased saliva flow rate, pH, total protein and calcium concentration during pregnancy, accompanied by increased α–amylase activity and sodium concentration. The study by Naveen et al., (2014) reported an increased salivary flow rate in pregnant women and attributed the increase in flow rate to elevated estrogen and progesterone levels, while the decrease in pH and buffer capacity was linked to reduced plasma $HCO3^-$ ion concentration and an increase in α–amylase concentration Lasisi and Ugwuadu, (2014). Found significantly reduced salivary pH, potassium, and bicarbonate concentrations, with elevated sodium and phosphate concentrations in pregnant women, but no significant difference in salivary flow rate, total protein, and calcium concentrations Hugoson et al., (2009), observed increased potassium and calcium concentrations in saliva during pregnancy with no correlation between salivary potassium concentration and

flow rate. These diverse findings underscore the multifaceted impact of pregnancy on salivary flow rate, reflecting a complex interplay that warrants further investigation.

The results of our study revealed a significant decrease in specific gravity among pregnant animals compared to their non-pregnant counterparts. Simultaneously, although the density exhibited a numerical decrease in pregnant animals, this change did not attain a statistical significance. These findings show a potential interdependence between specific gravity and density. Specific gravity and density are related physical properties. The observed decrease in specific gravity suggests alteration in the composition of saliva, potentially influenced by hormonal fluctuation during pregnancy. The decrease in the specific gravity may be reflective of variations of concentrations of solute in saliva. The non-significant numerical decrease is still of biological significance. However, to the best of our knowledge, there is a notable gap in the existing literature addressing the impact of early pregnancy on specific gravity and density of saliva in bovine species. Further investigations are required to explore the physiological aspects of pregnancy affecting the specific gravity and density and to address the existing gap in knowledge.

The findings of our study reveal a significant decrease in the electrical conductivity of saliva in pregnant animals. The electrical conductance in solutions is affected by the degree of dissociation of electrolytes molecules, the interaction of ions with one another and with the solvent and the inter- ionic forces. In the case of saliva, it has been reported that the concentration of electrolytes and their interactions are regulated by fluctuating hormone levels.

To explain the reason for decreased electrical conductance, we must investigate into the physiological aspects of bovine pregnancy. Bovine pregnancy is a stage of gestational di-estrus characterized by elevated levels of progesterone and lower levels of estrogen. Scientific investigations have reported the favorable effects of estrogen on the electrical conductance of mucus and nerve cells. For example, Platt et al., (1967) reported high electrical conductance in cervical mucus when levels of estrogen were higher during normal menstrual cycle. On the other hand, reduced electrical conductivity was recorded when progesterone levels were high.

Usaomer et al., (2011) carried out the electrophysiological study to explore variations in the electrical conduction of nerve during three stages of menstrual cycle. The three stages were early follicular phase (EFP), mid luteal phase (MFP) and late follicular phase (LFP), characterized by low levels of estrogen and progesterone, higher levels of progesterone, and high estrogen and low progesterone levels respectively. Significant differences (0.004) were found in the electrical conductance of nerve (NCS= nerve conduction study) between EFP and LFP, while statistically non –significant differences were found between EPF and MLP. The increased electrical nerve conduction in LFP was correlated with elevated level of estrogen.

Azarmina et al., (2011) reported that progesterone caused neuroinhibition of optic nerve conduction. Similarly, Yilmaz et al., (1998), reported the neuroexcitatory and neuroinhibitory potentials of estrogen and progesterone respectively on visual pathways Nakagawa et al., (2005), studied the comparative electrophysiological aspects of heart and reported variations in the features of electrocardiogram (ECG) of male and female. The sex-based differences in the features of ECG were correlated with the levels of estrogen in female.

Based on the preceding discussion one can infer that the electrical conductivity in a medium can be affected by relative concentrations of estrogen and progesterone. In our study, the lower electrical conductivity in diluted and undiluted saliva is attributed to increased concentration of progesterone associated with pregnancy. These results align with findings of the researchers mentioned earlier, reinforcing the significant impact of hormonal concentrations on electrical conductivity in our experimental context.

Crystallization of saliva has emerged as an interesting silometric procedure capturing the attention of researchers dedicated to uncovering specific non-invasive point-of-care biomarkers for the exploration and monitoring of reproductive processes in both animals and humans. In our study, we observed six different patterns of crystallization ranging from none to dotted, fern like, fir like, branch like and various combinations of these patterns. The prevalence of these patterns differed significantly between PA and NPA groups. In PA group, the predominant crystallization pattern was fern-like (26.19%) followed by branch+fern (19.4%) pattern. On the other hand, the NPA group revealed different spectrum of crystallization. The branch+fir pattern took the lead (29.03%), followed by branch+fern (22.58%), branch+fern+fir (19.35%) and dot-like (19.35%). In short, the fern-like crystallization pattern was predominant (26.19%) in PA, while branch+fir pattern emerged as predominant pattern in NPA. In a recent study by Chavan et al., (2023), the salivary crystallization patterns Zebu cows during estrus and pregnancy were investigated and compared. Six distinct type of crystallization patterns, including branch like, fir like and fern like and various combination of these identified. Contrasting to our study, the fern-like crystallization pattern was observed on the 16th day of the estrous cycle and on the day of estrus, where the branch-fir like pattern predominated in pregnant animals.

The variations in the patterns of crystallization encourage the researchers to deeply investigate in to the biochemical and hormonal factors influencing the patterns. This further encourages the broader implications

of saliva crystallization to monitor the reproductive processes in animals and humans. The study by Barbato et al., (1993), reported that the fertile phase in 88% of the menstrual cycles could be marked by the appearance of crystallization Guida et al., (1993), reported that salivary ferning can be used for determining the fertile period. They concluded that salivary crystallization was influenced by estrogen, catecholestrogen and opioid positively. Further investigations and collaborations are warranted to validate and expand the diagnostic value of this non-invasive approach in monitoring the reproductive processes in diverse populations.

## 4. Conclusion

The study aimed to discover potential early pregnancy biomarkers in cattle through saliva analysis, revealing variations in pH, buffering capacity, and other properties between pregnant and non-pregnant cows. Increased pH and decreased buffering capacity in pregnant cows suggest a biological response to early pregnancy, while numerical decreases in salivary flow rate indicate complex pregnancy effects. Significant changes in specific gravity and electrical conductivity, along with unique crystallization patterns, highlight the need for further research to validate diagnostic value and explore broader applications in diverse populations. The study suggests exploring various physical aspects of saliva, such as pH, flow rate, and electrical conductivity, for a non-invasive and cost-effective pregnancy diagnosis model in cattle, with potential applications in veterinary, biomedical, and wildlife science. Significant changes in salivary parameters were observed between pregnant and non-pregnant cows from day 25 to 35, emphasizing the potential of saliva for early pregnancy diagnosis and reproductive efficiency in dairy farm management. However, further research is needed to establish physiological ranges and validate findings for broader application.

**Conflict of interests:**
The author(s) declared no potential conflicts of interest concerning research, authorship, and/or publication with the work submitted.

## References


1. Alemrajabi, M.S., Dadkhah, A., Kasayizadegan-Mahabadi, S.A. and Sadrzadeh-Afshar, M.S., 2021. Serum and Salivary Levels of Glucose and Urea in Pregnant and Non-pregnant Women: A Comparative Study. Disease and Diagnosis, 10(4), pp.164-168.
2. Al–Nuaimy, K.M. and Al–Doski, F.S., 2003. Pregnancy–related changes in oral health and human unstimulated whole saliva. Al-Rafidain Dental Journal,3(2), pp.108-115.https://doi.org/10.33899/RDEN.2003.165765.
3. Azarmina, M., Soheilian, M. and Azarmina, H., 2011. Increased latency of visual evoked potentials in healthy women during menstruation. Journal of ophthalmic & vision research, 6(3), p.183..https://doi.org/10 .3389/fped.2022.938139.
4. Bakhshi, M., Sabet, M.S., Hashemi, E.S., Bakhtiari, S., Tofangchiha, M., Marhabi, S.A. and Alirezaei, S., 2012. Evaluation of biochemical changes in unstimulated salivary, calcium, phosphorous and total protein during pregnancy. African Journal of Biotechnology, 11(8), pp.2078-2083.
5. Balhara, A.K., Gupta, M., Singh, S., Mohanty, A.K. and Singh, I., 2013. Early pregnancy diagnosis in bovines: current status and future directions. The Scientific World Journal, 2013.https://doi.org/10.1155/2013/958540.
6. Barak, S., Oettinger-Barak, O., Oettinger, M., Machtei, E.E., Peled, M. and Ohel, G., 2003. Common oral manifestations during pregnancy: a review. Obstetrical & gynecological survey, 58(9), pp.624-628.https://doi.org/10.97/01.OGX.0000083542.14439.CF.
7. Berardono, B., Melani, D., Ranaldi, F., Giachetti, E. and Vanni, P., 1993. Is the salivary "ferning" a reliable index of the fertile period. Acta Eur Fertil, 24(2), pp.61-65.https://doi.org/10.1016/s0010-7824(98)000390.
8. Chavan, N.B., Kumaresan, A., Chhillar, S., Nayak, S., Prakash, M.A., Lathika, S., Baithalu, R.K., Onteru, S., Manimaran, A. and Kimothi, S.P., 2023. Salivary crystallization pattern: a possible unconventional tool for the timing of insemination and early pregnancy diagnosis in zebu cows. Journal of Dairy Research, 90(1), pp.21-25.https://doi.org/10.1017/S0022029922000887.
9. Devi, I., Singh, P., Lathwal, S.S., Kumaresan, A. and Dudi, K., 2016. Evaluation of salivary electrolytes during estrous cycle in Murrah buffaloes with reference to estrus detection. Veterinary World, 9(10), p.1157.https://doi.org/10.14202/vetworld.2016.1157-1161

10. Guida, M., Barbato, M., Bruno, P., Lauro, G. and Lampariello, C., 1993. Salivary ferning and the menstrual cycle in women. Clinical and experimental obstetrics & gynecology, 20(1), pp.48-54.
11. Hegde, S., Thakur, N.S., Kohli, S., Shukla, V., Siddiqui, A., Patel, P. and Payasi, S., 2016. A comparative evaluation of salivary flow rate, pH, buffering capacity, calcium and total protein levels in pregnant and non pregnant women. Journal of advanced medical and dental sciences research, 4(4), p.92.https://doi.org/10.21276/jamdsr.2016.4.4.20.
12. Hugoson, A., 1972. Salivary secretion in pregnancy a longitudinal study of flow rate, total protein, sodium, potassium and calcium concentration in parotid saliva from pregnant women. Acta Odontologica Scandinavica, 30(1), pp.49-66.https://doi.org/103109/0001635/209004592.
13. Karnik, A.A., Pagare, S.S., Krishnamurthy, V., Vahanwala, S.P. and Waghmare, M., 2015. Determination of salivary flow rate, pH, and dental caries during pregnancy: A study. Journal of Indian Academy of Oral Medicine and Radiology, 27(3), pp.372-376..https://doi.org/10.4103/0972-1363.170454.
14. Kivelä, J., Laine, M., Parkkila, S. and Rajaniemi, H., 2003. Salivary carbonic anhydrase VI and its relation to salivary flow rate and buffer capacity in pregnant and non-pregnant women. Archives of oral biology, 48(8), pp.547-551..https://doi.org/10.1016/S0003-9969(03)00096-7.
15. Laine, M. and PienihaÈkkinen, K., 2000. Salivary buffer effect in relation to late pregnancy and postpartum. Acta Odontologica Scandinavica, 58(1), pp.8-10.https://doi.org/10.1080/000163500429361.
16. Lasisi, T.J., 2014. Pregnancy related changes in human salivary secretion and composition in a
17. Nigerian population. African Journal of Medicine and Medical Sciences, 43(4), pp.347-351.
18. LINFORD, E., 1974. Cervical mucus: an agent or a barrier to conception?. Reproduction, 37(1), pp.239-250.
19. Mekonnin, A., Howie, A.F., Riley, S., Gidey, G., Tegegne, D.T., Desta, G., Ashebir, G., Gebrekidan, B. and Harlow, C., 2017. Serum, milk, saliva and urine progesterone and estradiol profiles in crossbred (Zebu x Holstein Friesian) dairy cattle. Animal Husbandry, Dairy and Veterinary Science, 1(3).https://doi.org/10.15761/AHDVS.1000118.
20. Migliario, M., Bindi, M., Surico, D., De Pedrini, A. and Minsenti, S., 2021. Changes in salivary flow rate and pH in pregnancy. European Review for Medical & Pharmacological Sciences, 25(4).
21. Moriyoshi, m., tamaki, m., nakao, t. And kawata, k., 1996. Early pregnancy diagnosis in the sow by saliva progesterone measurement using a bovine milk progesterone qualitative test EIA kit. Journal of Veterinary Medical Science, 58(8), pp.737-741.https://doi.org/10.1292/jvms.58.737.
22. Nakagawa, M., Ooie, T., Ou, B., Ichinose, M., Takahashi, N., Hara, M., Yonemochi, H. and Saikawa, T., 2005. Gender differences in autonomic modulation of ventricular repolarization in humans. Journal of Cardiovascular Electrophysiology, 16(3), pp.278-284.https://doi.org/10.1046/j.1540-8167.2005.40455.x.
23. Naveen, S., Asha, M.L., Shubha, G., Bajoria, A.A. and Jose, A.A., 2014. Salivary flow rate, pH and buffering capacity in pregnant and non-pregnant women–a comparative study. JMED research, 2014(2014), pp.1-7.https://doi.org/10.5171/2014.506946.
24. Platt, H.A., Connell, E.B. and Stone, M.L., 1968. Conductivity of cervical mucus during the menstrual cycle and pregnancy. Fertility and Sterility, 19(1), pp.85-90.https://doi.org/10.1016/S0015-0282(16)36547-5.
25. Ravinder, R., Kaipa, O., Baddela, V.S., Sinha, E.S., Singh, P., Nayan, V., Velagala, C.S.N., Baithalu, R.K., Onteru, S.K. and Singh, D., 2016. Saliva ferning, an unorthodox estrus detection method in water buffaloes (Bubalus bubalis). Theriogenology, 86(5), pp.1147-1155.https://doi.org/10.1016/j.theriogenology.2016.04.004.
26. Rockenbach, M.I., Marinho, S.A., Veeck, E.B., Lindemann, L. and Shinkai, R.S., 2006. Salivary flow rate, pH, and concentrations of calcium, phosphate, and sIgA in Brazilian pregnant and non-pregnant women. Head & face medicine, 2, pp.1-5.https://doi.org/10.1186/s13012-020-01041-8.
27. Saluja, P., Shetty, V., Dave, A., Arora, M., Hans, V. and Madan, A., 2014. Comparative evaluation of the effect of menstruation, pregnancy and menopause on salivary flow rate, pH and gustatory function. Journal of clinical and diagnostic research: JCDR, 8(10), p.ZC81. https://doi.org/10 .7860/JCDR/2014/9935.5071.
28. Skalova, I., Fedorova, T. and Brandlova, K., 2013. Saliva crystallization in cattle: a new possibility for early pregnancy diagnosis? Agricultura tropica et subtropica, 46(3), pp.102-104.https://doi.org/10.2478/ats-2013-0018.
29. Soma-Pillay, P., Nelson-Piercy, C., Tolppanen, H. and Mebazaa, A., 2016. Physiological changes in pregnancy: review articles. Cardiovascular journal of Africa, 27(2), pp.89-94.https://doi.org/10.5830/CVJA-2016-021.

30. Ustaömer, K., Sözay, S. and Sarıfakıoğlu, B., 2021. Effect of estrogen and progesterone on nerve conduction studies during ovarian cycle. Turkish journal of physical medicine and rehabilitation, 67(4), p.502.https://doi.org/ 10.5606/tftrd.2021.6628
31. Warning, J.C., McCracken, S.A. and Morris, J.M., 2011. A balancing act: mechanisms by which the fetus avoids rejection by the maternal immune system. Reproduction, 141(6), p.715.https://doi.org/ 10.1530/REP-10-0360.
32. Wong, D.T., 2006. Salivary diagnostics powered by nanotechnologies, proteomics and genomics. The Journal of the American Dental Association, 137(3), pp.313-321.https://doi.org/ 10.14219/jada.archive.2006.0180.
33. Yılmaz, H., Erkin, E.F., Mavioğlu, H. and Sungurtekin, Ü., 1998. Changes in pattern reversal evoked potentials during the menstrual cycle. International Ophthalmology, 22, pp.27-30.https://doi.org/ 10.1023/a:1006165126702.
34. Yoshizawa, J.M., Schafer, C.A., Schafer, J.J., Farrell, J.J., Paster, B.J. and Wong, D.T., 2013. Salivary biomarkers: toward future clinical and diagnostic utilities. Clinical microbiology reviews, 26(4), pp.781-791.https://doi.org/ 10.1128/CMR.00021-13
35. Yousefi, M., Parvaie, P. and Riahi, S.M., 2020. Salivary factors related to caries in pregnancy: A systematic review and meta-analysis. The Journal of the American Dental Association, 151(8), pp.576-588.https://doi.org/ 10.1016/j.aime.2020.04.021Contreras-Aguilar, M.D., Vallejo-Mateo, P.J., Lamy, E., Escribano, D., Cerón, J.J., Tecles, F. and Rubio, C.P., 2021. Changes in saliva analytes in dairy cows during peripartum: a pilot study. Animals, 11(3), p.749.Doi:org/10.3390/ani11030749.
36. Kivelä, J., Laine, M., Parkkila, S. and Rajaniemi, H., 2003. Salivary carbonic anhydrase VI and its relation to salivary flow rate and buffer capacity in pregnant and non-pregnant women. Archives of oral biology, 48(8), pp.547-551.Doi.org/10.1016/S0003-9969(03)00096-7.
37. Brown, T. L., LeMay, H. E., Bursten, B. E., Murphy, C. J., & Woodward, P. M. (1969). Chemistry: The Central Science (Vol. 12). Prentice Hall.
38. Skalova, I., Fedorova, T. and Brandlova, K., 2013. Saliva crystallization in cattle: a new possibility for early pregnancy diagnosis? Agricultura tropica et subtropica, 46(3), pp.102-104.DOI: 10.2478/ats-2013-0018.

**TABLE 1.** Comparative result of the parameters of physical properties of saliva of pregnant and non-pregnant cows

| ***Parameter*** | **Non-Pregnant Animal** | **Pregnant Animal** | **P-value** |
|---|---|---|---|
| *Buffer capacity* | 7.40 ± 0.10 | 7.36 ±0.30 | 0.885 |
| *Flow-rate mg/min* | 91.92±1.13 | 91.75±0.81 | 0.932 |
| *Specific gravity S/min* | 0.000173±0.00 | 0.000146±0.00 | 0.049 |
| *pH* | 8.133 ± 0.13 | 9.325 ± 0.13 | 0.001 |
| *Density g/ml* | 0.173 ± 0.005 | 0.173 ± 0.005 | 0.835 |
| *Conductivity-diluted* | 0.666 ± 0.029 | 0.5385 ± 0.028 | 0.003 |
| *Conductivity-undiluted* | 3.00 ± 0.19 | 2.42 ± 151 | 0.020 |

**TABLE 2.** Comparison pattern of saliva crystallization (%) of pregnant and non-pregnant cows

| **Pattern** | **Pregnant** | **Non-pregnant** |
|---|---|---|
| Fern like | 26.19 | 0.00 |
| Fir like | 4.76 | 6.45 |
| Branch like | 11.90 | 0.00 |
| Dot like | 9.52 | 19.35 |
| Branch+Fir | 7.14 | 29.03 |
| Branch+Fern+Fir | 4.76 | 19.35 |
| Branch+Fern | 19.04 | 22.58 |
| Fern+Fir | 7.14 | 0.00 |
| None | 9.52 | 3.22 |

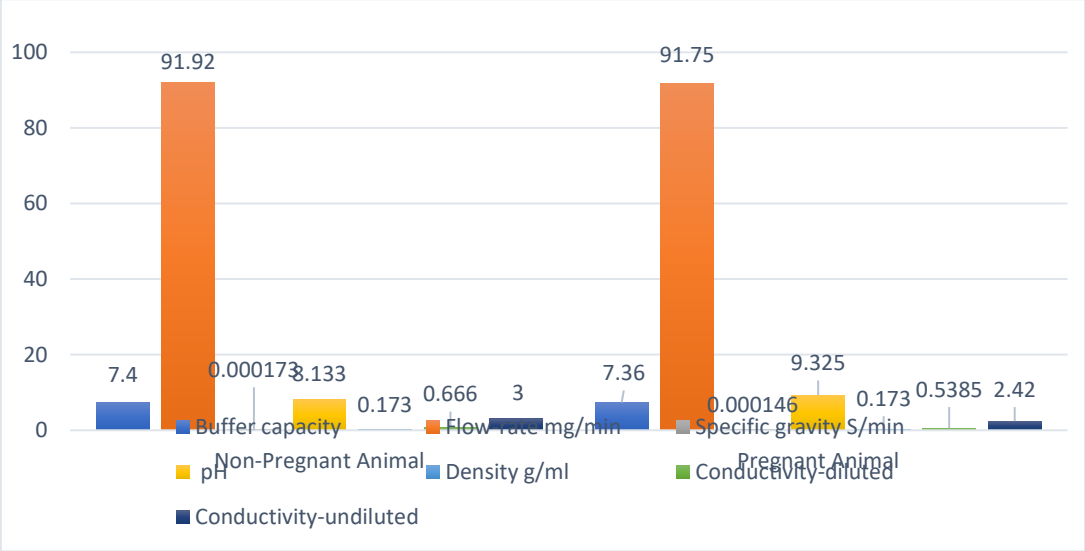


**FIGURE 1.** Showing comparative result of the parameters of physical properties of saliva of pregnant and non-pregnant cows

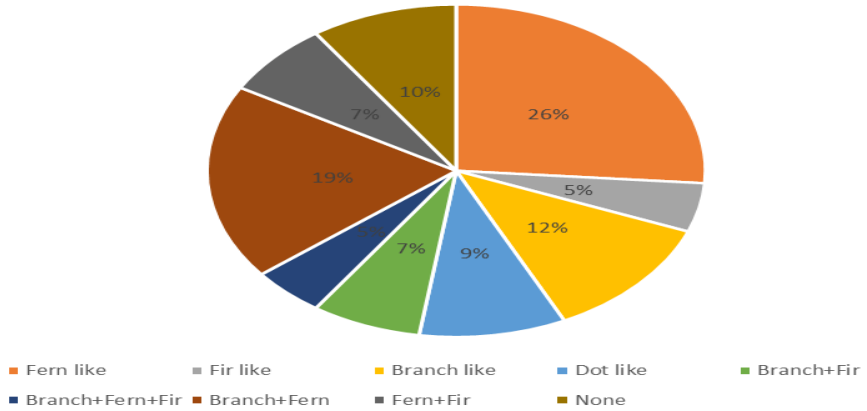


**FIGURE 2.** Showing comparison pattern of saliva crystallization (%) of pregnant and non-pregnant cow.