Salivary Crystallization and miRNA: Potential Biomarkers for Estrus Identification in Buffaloes

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Abstract

Estrus detection and properly timed insemination in buffaloes remains as a challenge, especially under smallholder production systems, wherein application of automation and sensor-based tools for reproduction management is not possible. Traditionally, manual observation of animals for the signs of estrus detection is being practiced in developing countries, which is labor intensive and often results in reduced estrus detection efficiency and accuracy, as a majority of the buffalo shows estrus onset/behavior during night or early morning hours. Further, not all buffaloes show overt estrus signs so that they can be visually observed. The incidence of silent estrus is very high in buffaloes, and, estrus detection is really a challenge in this species especially during low-breeding season. In the recent past, several attempts have been made to identify biomarkers in body fluids and to develop cow-side test for estrus detection. In this chapter, the use of salivary crystallization patterns and salivary molecules as an aid for estrus detection is detailed.

Keywords

Estrus detection · Salivary fern pattern · Buffaloes · miRNAs · Fertility

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6.1 Introduction

Estrus detection needs further efficient methods for buffaloes compared to other domestic animals because of their short estrus duration (5–27 h) and absence of clear estrus behavior. Statistically, only 50% of the estrus or heat symptoms in buffaloes are detected with 41% estrus detection efficiency. This leads to the improper insemination timings in both the organized farms (20.75%) and field (30%) conditions (Srivastava et al. 2013). In India alone, around 13.62 million buffaloes (25%) out of 54.48 million buffaloes are improperly detected for estrus symptoms. A similar scenario can also be expected in other developing countries. One missing estrus in buffaloes causes an estimated loss of INR. 5901 to 7728 to the buffalo farmers (Abdullah et al. 2014), which subsequently leads to nearly INR 45000 million loss to the Indian economy alone. Therefore, efficient and easy estrus identification is essential for buffalo farmers. There are many different methods available for detection of heat in domestic livestock, such as chalk tail head (Macmillan and Curnow 1977), continuous videotaping (Donaldson et al. 1968), visual observation (Williamson et al. 1972), pressure sensation device (Shipka 2000), pedometers (Arney et al. 1994), and many more. Among them, pedometers and plasma progesterone detection (Delwiche et al. 2001) appear to have 100% efficiency in heat detection. However, majority of all these heat-detecting methods are not studied properly in buffaloes. Moreover, these methods require skilled personnel, which is impractical in rural countries like India. Hence, there is an urgent need of accurate, most efficient, reliable, and field applicable heat-detecting method for buffaloes.

To overcome the heat or estrus detection problem in buffaloes, researchers are trying to explore biomarkers in noninvasive fluids. The noninvasive fluids include milk, urine, cervico-vaginal fluid, feces, and saliva, which are the greater resources of biomarkers. Among these fluids, saliva is available every time irrespective of any physiological stage. In addition, its collection is cost effective (Yoshizawa et al. 2013), and it appears to have long period of stability (Ang et al. 2011). Therefore, the saliva is one of the ideal biofluids for the discovery of biomarkers for estrus in buffaloes. Particularly, the cell-free saliva is better to represent the physiological status of animals as it is a combination of partial ultrafiltrate of the plasma as well as the secretion of salivary glands.

The biomarkers might be mi-RNA/small-RNA, carbohydrates, proteins, lipids, steroids, nucleic acids, ions, and physiochemical/morphological characteristics like crystallization pattern formations of body fluids. Any of these biomolecules and morphological characteristics specifically associated with estrus can be served as biomarkers for estrus detection. In the recent times, the detection of miRNA in major body fluids gained importance, since it reflects the various physiological conditions of the animal. In addition, the sensitivity and specificity of RNA-based biomarkers are better than protein biomarkers. Further, the cost of RNA biomarker detection is cheaper than protein biomarker detection as RNA-based biomarker can be detected by field applicable RT-LAMP (Reverse-transcriptase loop-mediated isothermal

amplification) technology without the synthesis of antibodies, which are needed for a protein biomarker (Xi et al. 2017). On the other hand, physical patterns like ferning/crystallization pattern of the biofluids serve as a major indicator of animal reproductive status. Therefore, by quantifying the salivary miRNA and characterization of fern patterns, it is possible to develop an estrus identification method in buffaloes.

6.2 Salivary Crystallization

Crystallization of body fluid was first described by Papanicolaou (1946) in vaginocervical mucus of women. Garm and Skjerven (1952) noticed the cervico-vaginal crystallization of cows and reported that during estrus, the fern morphology crystals appeared, and they disappeared during the luteal phase of the estrous cycle. Crystallization was also described in nasal mucus (Peterson 1984), tears (Golding and Brennan 1989), milk or colostrum (Zondek and Rozin 1954), and saliva (Pardo-Carmona et al. 2010). Among these fluids, saliva crystallization is easy to perform. In the recent years, monitoring of menstrual cycle in women was carried out by observing salivary fern patterns. To observe the salivary fern patterns by women themselves, different kinds of small hand-held microscopes or pocket microscopes or paper microscopes are currently available in the market. These pocket microscopes are giving an average accuracy of 92% (Guida et al. 1993). In the recent years, the salivary crystallization method is also used to determine the ideal mating time in bitches (Pardo-Carmona et al. 2010), monitoring of menstrual cycle in wild female Bornean orangutan (Kubatova and Fedorova 2016) and in cattle for determining early pregnancy diagnosis after AI (Skalova et al. 2013) as well as for determining estrus time (Gnanamuthu and Rameshkumar 2015). The researchers hypothesized that estrogen has positive influence on salivary crystallization. However, the exact mechanism for the appearance of typical fern pattern is not yet clear.

The salivary crystallization method was also used for the first time to determine the estrus stages in buffaloes in our lab (Ravinder et al. 2016). The salivary fern patterns were observed throughout the estrus cycle of buffaloes, and fern patterns were also quantified by fractal analysis method. In this study, a total of 450 saliva samples were collected from 8 female nonpregnant Murrah buffaloes for two purposes: First, to prepare a smear on glass slides by using a standard blood smear technique for observing different types of saliva crystallization or ferning patterns; second, to estimate the salivary estradiol and progesterone concentrations. The smears showed different crystallization patterns throughout the estrus cycle, and they are categorized into typical symmetrical fern-like, branch-like, fir-like, combination of fir-fern branch, dotted, and nontype (Figs. 6.1 and 6.2). The typical symmetrical fern-like pattern appeared during the estrus stage with a proportion of 0.84 ($P < 0.01$), which was a higher proportion than the general proportion of estrus detection (0.50/50%) in buffaloes in the field condition. The salivary levels of total estradiol and E2/P4 ratio were found to be higher ($P < 0.05$) at the estrus stage than

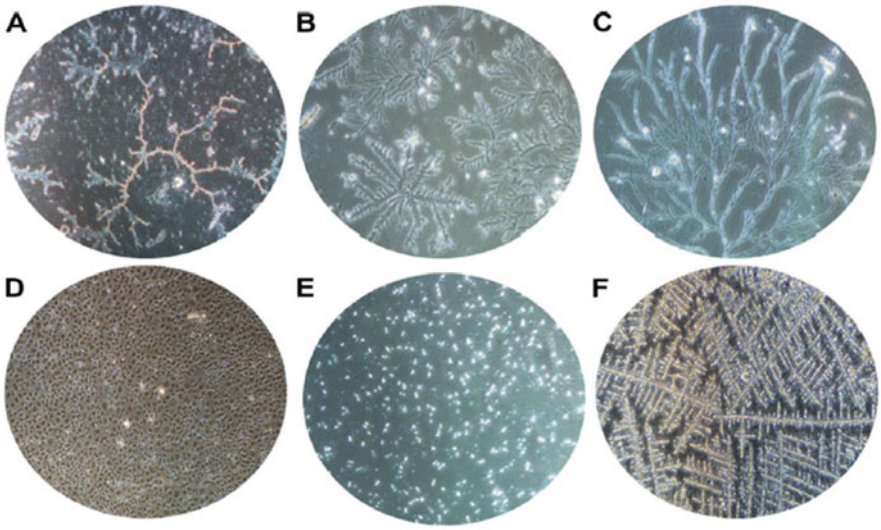

Fig. 6.1 Different salivary crystallization patterns in buffaloes. Branch-like (**a**), fern-like (**b**), fir-like (**c**), none (**d**), dotted (**e**), and typical fern patterns (**f**). (This image was taken from Ravinder et al. 2016 for educational purpose)

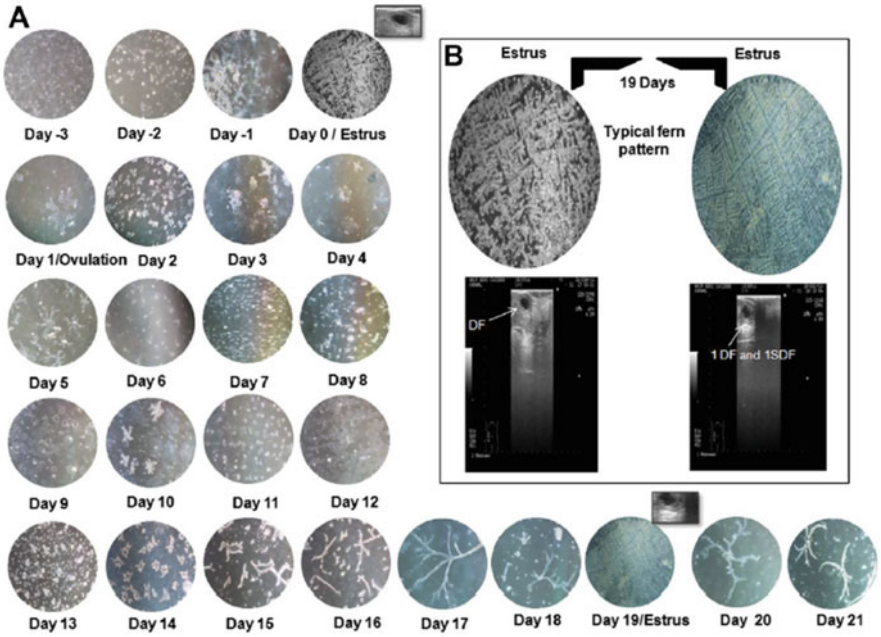

Fig. 6.2 Salivary fern patterns during estrus cycle of buffaloes along with the ultrasound images of ovaries. (This image was taken from Ravinder et al. 2016 for education purpose)

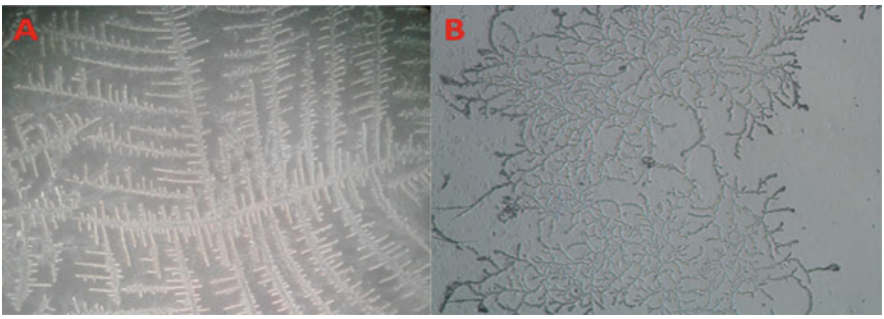

Fig. 6.3 (**a**) Estrus and (**b**) Diestrous stages. The day 10 of the estrous cycle was considered as the diestrous stage. (This image was taken from Surla et al. 2021 of for educational purpose)

the diestrus stage, and the salivary progesterone levels were significantly higher at the diestrus stage than the estrus stage (Fig. 6.3). On the basis of the above findings, it can be concluded that the higher levels of estradiol during estrus stage cause the typical fern-like crystallization patterns of the saliva in buffaloes and it can be applied in the field condition for determining the estrus in buffaloes.

The saliva crystallization pattern method was later validated in more than 500 estrus events (Surla et al. 2021). In this study, 4 buffaloes population samples were considered: organized herd (PS1), buffaloes came for AI (PS2), buffaloes with induced estrus by PGF2α (PS3), and random buffaloes at farmers doorsteps (PS4). In the PS1, ten buffaloes were exclusively studied for this research. From this PS1, saliva samples were collected from 149 potential estrus stages over a period of one year, in which 111 samples showed typical fern-like patterns before 8–12 h of the actual estrus behavioral sings. In the PS2, a total of 114 buffaloes were observed for salivary fern patterns, which were brought for AI centers. Among them 44 buffaloes confirmed for typical fern patterns in which 15 buffaloes were found to be pregnant after AI and 38 buffaloes did not show any typical fern patterns, but still 24 buffaloes became pregnant after AI. In the PS3, 44 buffaloes were induced for estrus by administering a single dose of PGF2α in which only 7 buffaloes were found to show typical fern-like crystallization patterns. In the last population sample, PS4, a total of 275 buffaloes with unknown reproductive history and absence of estrus sings were considered and the typical fern-like crystallization patterns were observed by using a paper-based microscope called Foldscope (Fig. 6.4). Among them, only 22 buffaloes have shown typical fern patterns, and 20 of the buffaloes were confirmed for estrus by veterinarians. These data and observations, and a significant higher proportion of estrus identification in the PS1 and PS4 on the basis of salivary fern-like patterns, further support the utility of saliva crystallization-based estrus identification in the field conditions: farmers are trained for the detection of saliva crystallization patterns by using a simple filed applicable microscope.

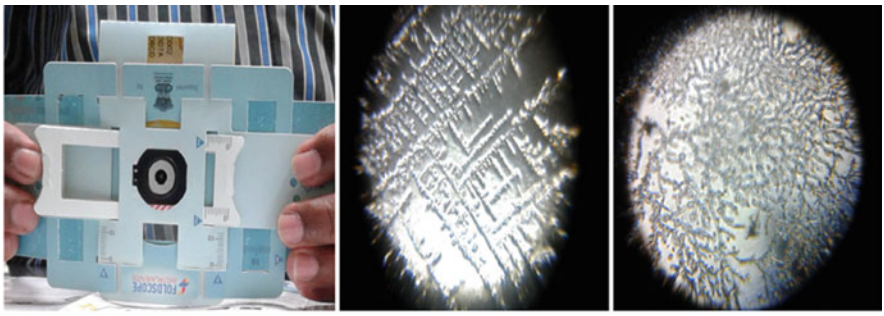

Fig. 6.4 Salivary fern patterns of buffaloes by the Foldscope. (**a**) Foldscope. (**b**) Estrus stage (**c**) Diestrous stages. The day 10 of the estrous cycle was considered as the diestrous stage (This image is taken from the journal reproductive biology for educational purpose)

6.3 Chemical Composition of the Saliva Fern Patterns

The mucus/tear/saliva forms fern-leaf-like patterns when they are allowed to dry on a clean and smooth surface, like a glass slide. The mechanism behind the ferning is still a little mystery for researchers. Ferning patterns in tears were extensively studied in humans to address various eye problems. Many hypotheses have been put forward to explain the ferning or crystallization. Golding and Brenan (1989) hypothesized that ferning is not dependent on any single chemical species but the interrelationship of many different chemical species. Vaikoussis et al. (1994) suggested that the balance between sodium chloride and mucus is important for ferning. According to Kogbe et al. (1991), for successful ferning, the ratio between divalent calcium and magnesium ions to monovalent sodium and potassium ions plays a major role. Pearce and Tomlison (2000) studied the chemical composition of human tear ferns and analyzed the locations of chemical elements in the fern. When tear fern patterns were examined under a scanning electron microscope, two main structures were observed: dendrites and cubical crystals. Dendrites make up a major part of the fern, whereas cubical crystals are present adjacent to the dendrites. X-ray analysis of the dendrites and cubical crystals structures showed that the dendrites are made up of NaCl, along with other ions, and cubical crystals are made up of KCl. In this study, sulfur was also detected at the peripheral region of the tear smears, which suggests the possibility of presence of macromolecules like mucins and proteins at the periphery of the smears. Further, it was inferred that these macromolecules deposited at the very periphery of the dry teardrop may play a role in retardation of fern growth (Golding et al. 1994). These results propose that the macromolecules such as glycoproteins proteins and mucins may have some role in fern formation. This was the first study regarding the spatial location of organic (sulfur-containing) molecules in the fern. Such studies on buffalo saliva fern patterns are underway.

6.4 Salivary Mucins/Glycoproteins and Electrolytes

The appearance of typical fern-like patterns at the estrus stage is mainly influenced by increased estrogen levels. However, the exact mechanism for the appearance of typical fern pattern is not known. One of the causes for typical fern-like crystallization patterns of saliva could be salivary mucins and their associated carbohydrates and salts. Mucins are high-molecular-weight proteins composed of protein core and carbohydrate side chain (50–80% CHO glyco-conjugated). In human saliva, higher-molecular-weight mucins account for about 30% of total salivary mucins containing 12–15% of protein, 80–85% carbohydrates like sialic acid, fucose, etc. It is clear that mucins are majorly made up of large protein polypeptides and oligosaccharides side chains (Wu et al. 1994). However, specific mucins and their related carbohydrates and salts associated with typical salivary fern patterns at the estrus stage of buffaloes were not yet delineated. Identifying these molecules will help in developing a saliva-based color reaction for estrus identification in buffaloes.

The effect of hormones like estrogen and progesterone on electrolyte levels was well studied in humans. The concentrations of sodium, potassium, and chloride ions were observed to increase in parallel with the estrogen level during the ovulatory phase. The salivary ferning is formed by NaCl, which rises under the effect of estrogen (Alagendran et al. 2013) and decreases with progesterone (Macdonald 1969; Linford 1974). This observation proves that these salts along with hormones help in fern forming and brings out the day of fertile period in women (Pattanasuttinont et al. 2007). A study conducted by Dadlani et al. (1982) found that the same kind of changes occurs in the concentration of electrolytes corresponding to hormonal levels in serum during the menstrual cycle. It can be understood that the ovarian hormones have a good connection with electrolytes, which can be used for the prediction of ovulation as a noninvasive method.

Devi et al. (2016) quantified electrolytes concentration in saliva of Murrah buffaloes during estrus stage. They found that the concentration of calcium (8.76 ± 0.08–12.11 ± 0.11 mg/dl), inorganic phosphorus (6.56 ± 0.13–14.72 ± 4.50 mg/dl), magnesium (2.27 ± 0.14–5.79 ± 0.15 mg/dl), sodium (139.47 ± 0.31–159.62 ± 1.22 mmol/L), potassium (12.40 ± 0.22–-26.85 ± 1.22 mmol/L), and chloride (109.28 ± 0.41–137.07 ± 0.68 mmol/L) varied during the different phases of estrous cycle. However, during the estrus phase of the cycle, the concentration of all electrolytes was found to be significantly much higher compared to other phases of estrous cycle. This study found that the concentration of salivary electrolytes was positively associated with estrogen concentration and negatively associated with progesterone level.

6.5 Salivary miRNA

MicroRNAs are small (19–22 nucleotides), single-stranded RNA molecules, which regulate various biological process. They are synthesized in the nucleus of the cells in the presence of enzymes Drosha (RNA III endonuclease) and other

microprocessor complex proteins like DGCR8. The newly synthesized pre-miRNAs are then transported to cytoplasm by exprotein-5 and Ran-GTP. In the cytoplasm, pre-RNA is converted to mature miRNA by a Dicer complex. The mature miRNA participates in posttranscriptional regulation of gene expression either by repressing the translation or degradation of targeted mRNA molecules. In addition to intracellular function of miRNA within the cells from which they originate, they are secreted out of the cells through nanosecretory vesicles called exosomes.

miRNAs are omnipotent in all types of body fluids like serum, plasma, saliva, tear, and urine. When compared to other biomolecules like proteins and other RNAs, miRNAs are more stable in harsh conditions like low or high pH and they can be stored for long term. This property is because of their encapsulation within the lipoprotein complex called microvesicles or exosomes. Additionally, they are easy to detect using synthetic complementary oligonucleotides/PCR/DNA amplification. A large number of unique miRNAs like miR-182, miR450b-5p, miR-622, and many more have been identified in the saliva. Because of their stability, easy detection and most importantly their correlation with the different physio-pathological conditions, miRNAs can be used as biomarkers to monitor the health and reproductive physiology of farm animals.

Studies on salivary RNA-based biomarkers for estrus identification are very scanty in dairy animals. A study on buffaloes showed a suggestive higher abundance of heat shock protein 70 (*HSP70*) and toll-like receptor 4 (*TLR4*) transcripts in the buffalo saliva at the estrus stage than diestrus stage (Onteru et al. 2016). The study emphasized the usage of the direct saliva transcript analysis without RNA isolation. Such a technique along with the integration of RT-LAMP technology would be useful to be applicable for using RNA biomarkers in the field conditions for buffaloes. Toward this goal, a recent study (Singh et al. 2017) on the investigation of estrogen responsive miRNAs (*miR-24, miR-200c, miR-191, miR-16 and miR-223)* as estrus biomarkers in buffaloes' saliva indicated that the miR-16, miR-191, and miR-223 showed their higher abundance in the buffalo saliva on the day 6 and 18–19 than that of the estrus and tenth day (diestrus) of estrous cycle. This observation indicated that the lower presence of these miRNA in buffalo saliva on the estrus and tenth day of estrous cycle could intuitively indicate the presence of dominant follicle on the ovary. It should be noted that the lower expression of these miRNA in the ovarian follicles is essential to upregulate certain biochemical pathways, such as fatty acid biosynthesis and oocyte meiosis, and certain target genes like *FGF, BDNF*, *IGF1*, *KRAS, BCL2,* and *IGF1*, which are well known to be involved in the development of a dominant follicle on the ovary. Taken together, these clues further reinforce the utility of saliva RNA as biomarkers for ovarian physiology, thus the estrus stage in buffaloes. On the basis of these observations, further omics studies such as salivary transcriptome and miRNome are needed to identify the miRNA biomarker specific to the estrus stage in buffaloes. One of the bottlenecks for such omics studies is the high-quality RNA isolation itself. On the basis of our experience, the primary requisite to perform transcriptome studies from buffalo cell-free saliva is to develop an efficient RNA isolation method. If such an RNA isolation method is

developed, more reliable miRNA biomarkers can be identified from buffalo saliva for estrus detection.

6.6 Conclusion

Saliva is the one of the easily available noninvasive fluid every time to explore the biomarkers for estrus identification in buffaloes. Among several prospective biomarkers, observation of typical fern-like crystallization patterns of the dried salivary smear is a simple and potential estrus identification method in buffaloes. However, other potential biomarkers could be salivary miRNA specific to the estrus stage, but their search is still at a preliminary stage. The exploration of salivary miRNA specific to the estrus stage by transcriptome studies would be fruitful if any good method is devised for the isolation of high-quality RNA from the cell-free saliva of buffaloes.

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