

Research Article

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Salivary crystallization pattern: a possible unconventional tool for timing of insemination and early pregnancy diagnosis in zebu cows

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Abstract

The present study assessed if salivary crystallization pattern (ferning pattern formed as a result of the higher levels of salt content in the dried sample) could be used for estrus detection and for diagnosis of pregnancy/non-pregnancy in dairy cows. Saliva and blood samples were collected from non-pregnant cycling cows (Sahiwal breed; $n = 20$) on alternate days from the day of estrus till next estrus. Then, all the cows were inseminated and saliva and blood sampling were continued further for a period of 22 d post-insemination. Pregnancy diagnosis was carried out on day 45 post-insemination and eight cows were found to be pregnant. The salivary crystallization pattern and estradiol:progesterone ratio during estrous cycle and during pregnancy were compared among these cows. Six types of salivary crystallization patterns were discerned; distinct patterns such as branch-like, fern-like, fir-like and combinations of these. Fern-like pattern was observed in all the cows on the day of estrus (first measurement day) and furthermore, all of the cows that subsequently became pregnant had fern-like salivary crystallization pattern at the time of insemination. Saliva of all the pregnant cows showed branch-fir type of crystallization pattern on day 16 post-breeding while only 50% of non-pregnant cows showed this pattern on day 16 of estrous cycle. The appearance of fern-like pattern was positively and significantly related to estradiol:progesterone ratio ($r = 0.86$; $P < 0.001$). The findings were validated on a separate group of cycling cows ($n = 32$). We can conclude that salivary crystallization pattern might serve as a non-invasive and cost effective and easy-to-use cow-side tool for estrus detection and early pregnancy/non-pregnancy diagnosis in cows upon validation on a larger sample size.

Due to their good thermal tolerance and resistance to a large number of diseases, zebu cattle (*Bos indicus*) are extensively used in smallholder production systems in the tropics. However, the reproductive potential of the zebu cows is poor in comparison to that of *Bos taurus* breeds, which is a major constraint in realizing their full potential (Kanuya *et al.*, 2006). Proper estrus detection and identification of non-pregnant cows at the earliest possible day post-breeding is important for maintaining an optimum reproductive efficiency (Fricke, 2002). In zebu cattle, estrus duration can often be short and the overt signs of estrus weak, leading to difficulties in detecting estrus (Layek *et al.*, 2011). Automated estrus detection methods and decision support systems can be used in large-scale dairy farms but are prohibitively expensive for smallholder dairying. Similarly, for pregnancy diagnosis, progesterone assay in blood or milk and/or ultrasonography are used in large scale dairy farms but are not affordable for small farmers. Therefore, development of a non-invasive, easy to use and low-cost cow-side method for estrus detection and identification of pregnancy/non-pregnancy in dairy cows has long been the interest of researchers and farmers.

In cattle, the cervical mucus shows varying crystallization patterns on different days of the estrous cycle depending upon the concentrations of estrogen and progesterone, and is used as a tool to identify suitable time of insemination (Kumaresan *et al.*, 2001, 2009). Like cervical mucus, saliva also shows a crystallization pattern, which differs with different reproductive stages. Although information regarding crystallization patterns of ruminant saliva in relation to reproductive status is very limited, a few studies in cattle (Skalova *et al.*, 2013), buffalo (Ravinder *et al.*, 2016) and bitches (Pardo-Carmona, *et al.*, 2010) showed a significant relationship between crystallization pattern and reproductive status. Since the salivary crystallization pattern is reported to be influenced by the circulating concentrations of reproductive hormones (Alagendran *et al.*, 2010), we hypothesized that the salivary crystallization pattern

might vary with the stages of estrous cycle and during pregnancy in cows. Therefore, we assessed the changes in salivary crystallization pattern on different days of estrous cycle and during early pregnancy to find out if it could be used as a tool for timing of insemination and to diagnose early pregnancy in cows.

Materials and methods

Experimental plan

To study the salivary crystallization pattern during estrous cycle, saliva samples were collected from 20 cycling non-pregnant zebu cows (Sahiwal breed; parity 3–5) on alternate days starting from the day of estrus until next estrus (throughout the estrous cycle). Estrus detection was carried out twice a day (during evening and morning) by visual observation of cows for the signs of estrus (mucus discharge from vagina, mounting on other cows, standing to be mounted, swelling and reddening of vulva, bellowing, frequent urination, restlessness and rubbing against herd mates). Those cows identified to be in estrus were confirmed by rectal examination by an experienced veterinarian and also by using cervical mucus crystallization pattern. Then, all the cows were inseminated with cryopreserved semen from a proven bull, and saliva and blood samples were collected on alternate days from the day of insemination until day 22 post-breeding. Pregnancy diagnosis was carried out using ultrasonography (Aloka CO Ltd, Tokyo, Japan) on day 30–45 post-insemination. Out of the 20 cows inseminated, eight cows were found to be pregnant. The salivary crystallization pattern and estradiol:progesterone ratio of these eight cows during the estrous cycle and during pregnancy was compared. The study was approved by the Institute Animal Ethics Committee.

Saliva collection and assessment of crystallization pattern

On all occasions, saliva samples were collected in the morning before feeding the cows (7.00 AM). Manual collection (detailed in the online Supplementary File) was used to acquire around 10 mL of saliva which was centrifuged at 3000 rpm for 10 min to remove feed particles and debris, if any. The clear fraction of the supernatant was transferred to a microcentrifuge tube and 40 μ L of cell and dirt-free saliva was taken on a clean, grease-free glass slide and a thin smear was made using another glass slide. When the saliva was smeared evenly on the slide, it was allowed to air dry at room temperature (5–10 min) and was observed under microscope (200 \times ; Nikon Eclipse Ti-5, Tokyo, Japan) for crystallization pattern as per the procedure given by Ravinder *et al.* (2016). Larger, more robust and leaf-shaped crystals (elongated leaves similar to branches with long and well delimited stems) were considered as typical fern-like crystallization pattern. Other patterns with appearance like branches and fir were classified into branch like, fir like; branch and fir like; fir and fern like, and branch and fern like (Representative patterns are given in figure).

Blood collection and estimation of estradiol and progesterone concentrations

Peripheral blood was collected by jugular venipuncture and plasma was separated and stored in cryovials at -20°C until analysis. Estimation of plasma estradiol and progesterone was done using ELISA kits (USCN Life Science Inc., USA) according to

manufacturer's instructions. From the concentrations of estradiol and progesterone, the E2:P4 ratio was calculated.

Validation of experimental results

The major findings were validated using another set of 32 normal cyclic Sahiwal cows. These cows were inseminated during estrus using semen from a proven bull. The day of estrus was considered as day 0. Saliva samples were collected on every alternate day from day 0 to day 14 and on every day from day 15 to day 25. Pregnancy diagnosis was carried out on day 45 post-insemination. A total of 12 cows were found pregnant while 20 were found non-pregnant. The salivary crystallization pattern of pregnant and non-pregnant animals was compared.

Statistical analysis

Data obtained were analyzed statistically using *SigmaPlot* version 11.0 (SystatSoftware inc., USA) software. The frequency of appearance of salivary crystallization pattern in pregnant and non-pregnant cows (both in experimental and validation groups) was compared using Pearson's χ^2 test. Relationship of estradiol:progesterone ratio with salivary crystallization pattern was studied by Pearson Product Moment Correlation. The differences between expected proportion of a specific pattern and its actual observed proportion were analyzed using one proportion z-test using MedCalc easy-to-use statistical software (<http://www.medcalc.org/calc/test-one-proportion.php>).

Results

Saliva smears showed six types of crystallization patterns; distinct patterns such as branch-like, fern-like, fir-like and combinations of these patterns branch-fir, fir-fern and branch-fern (Fig. 1). Other bizarre patterns that did not fit into these classifications were classified into either atypical or none.

Salivary crystallization pattern during estrous cycle

The frequency of occurrence of different salivary crystallization patterns on different days of estrous cycle is depicted in Supplementary table S1. On the day of estrus, a clear fern-like pattern was observed in saliva of all the cows (difference from random, $P < 0.0001$; Figure 2). On day 2 and 4 of the estrous cycle, the dominant salivary crystallization pattern was the fir-like pattern (difference from random, $P < 0.01$) whereas the branch-like pattern was observed in the saliva of higher number of cows (difference from random, $P < 0.01$) on day 8 and 12 of the estrous cycle. Again, clear fern-like crystallization pattern was observed in saliva of all the cows on day 20 of the estrous cycle (subsequent estrus). Among the validation population also, all of the 12 cows that became pregnant showed typical fern-like salivary crystallization pattern on the day of insemination.

Salivary crystallization pattern during pregnancy

The frequency of occurrence of different salivary crystallization patterns on different days of pregnancy is depicted in Supplementary table S2. On the day of fertile estrus (insemination resulted in conception), saliva of all the cows showed a typical fern-like pattern (Fig. 2). The fern-like pattern continued to be dominant on day 2, 4 and 6 of pregnancy, which was not the

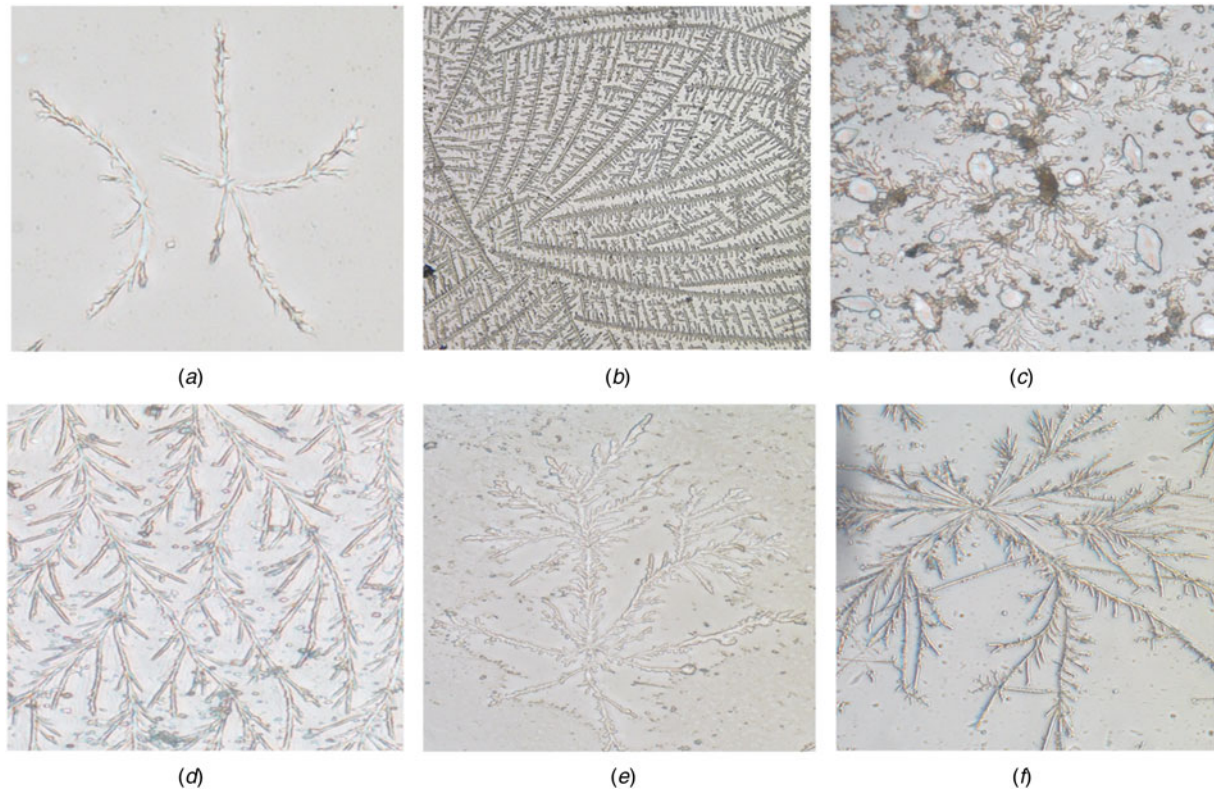


Fig. 1. Major crystallization patterns observed in saliva of cows; (a) branch like; (b) fern like; (c) fir like; (d) branch and fir like; (e) fir and fern like and (f) branch and fern like.

trend observed during the estrous cycle. Interestingly, on day 16 of pregnancy, saliva collected from all the pregnant cows showed only the branch-fir combination pattern (difference from random, $P < 0.0001$). Among the validation population, on day 16, 83.33% of pregnant cows showed branch and fir-like pattern while none of the non-pregnant cows showed this pattern. On day 20 of pregnancy, fir-fern pattern was dominant (difference from random, $P < 0.01$), which was not the trend observed during the estrous cycle. It was interesting to note that 87.5% of the pregnant cows did not show the fern-like pattern on day 20.

Relationship of estradiol:progesterone ratio with salivary crystallization

The peripheral concentrations of estradiol ranged from 24.3 to 60.1 pg/ml in Sahiwal cows (Supplementary figure S1). The progesterone concentrations ranged from 0.9 to 1.2 ng/ml on the day of estrus (Supplementary figure S2). It was observed that appearance of typical fern-like pattern was positively and significantly related to estradiol:progesterone ratio ($r = 0.86$; $P < 0.001$).

Discussion

In the present paper, we report the differences in salivary crystallization pattern during estrous cycle and pregnancy in zebu cows. Besides this, the relationship between estradiol:progesterone ratio and salivary crystallization pattern is also reported.

We observed that saliva of all the cows in both the experimental and validation populations showed a particular salivary crystallization pattern on the day of estrus. Further, it was also

observed that occurrence of this fern-like pattern was positively and significantly related to the estradiol:progesterone ratio. Earlier studies in dairy cattle also reported higher estradiol:progesterone concentrations on the day of estrus, which is required for events associated with standing estrus and ovulation of the oocyte (Pancarci *et al.*, 2002; Layek *et al.*, 2011). The relationship of estrogen:progesterone ratio with the appearance of typical fern-like salivary crystallization pattern is further supported by findings on day 20 post-insemination in non-pregnant cows. This is important, since otherwise the emergence of this pattern could theoretically have been a methodological error associated with the first sampling day. On day 20 post-insemination, all of the non-pregnant cows (returning to estrus) also showed this fern-like salivary crystallization pattern while the saliva of only one of the pregnant cows showed this pattern. Thus, the presence of this typical fern-like salivary crystallization pattern could be an indication of non-pregnancy in cows on day 20 after insemination. In women, it was reported that electrolyte concentrations in saliva samples increased significantly during the ovulatory phase but not during either the pre-ovulatory or the post-ovulatory period (Alagendran *et al.*, 2010). Therefore, it is possible that elevated estradiol concentrations might increase the salivary electrolyte concentrations and facilitate the appearance of the typical crystallization pattern in the saliva of cows on the day of estrus. Interestingly, all the cows that became pregnant had the salivary fern pattern on the day of insemination, supporting identification of estrus and the appropriate time for insemination of cows.

The branch- and fir-like salivary crystallization combination was found only during the progesterone-dominated phase. The differences between pregnancy and non-pregnancy, in terms of

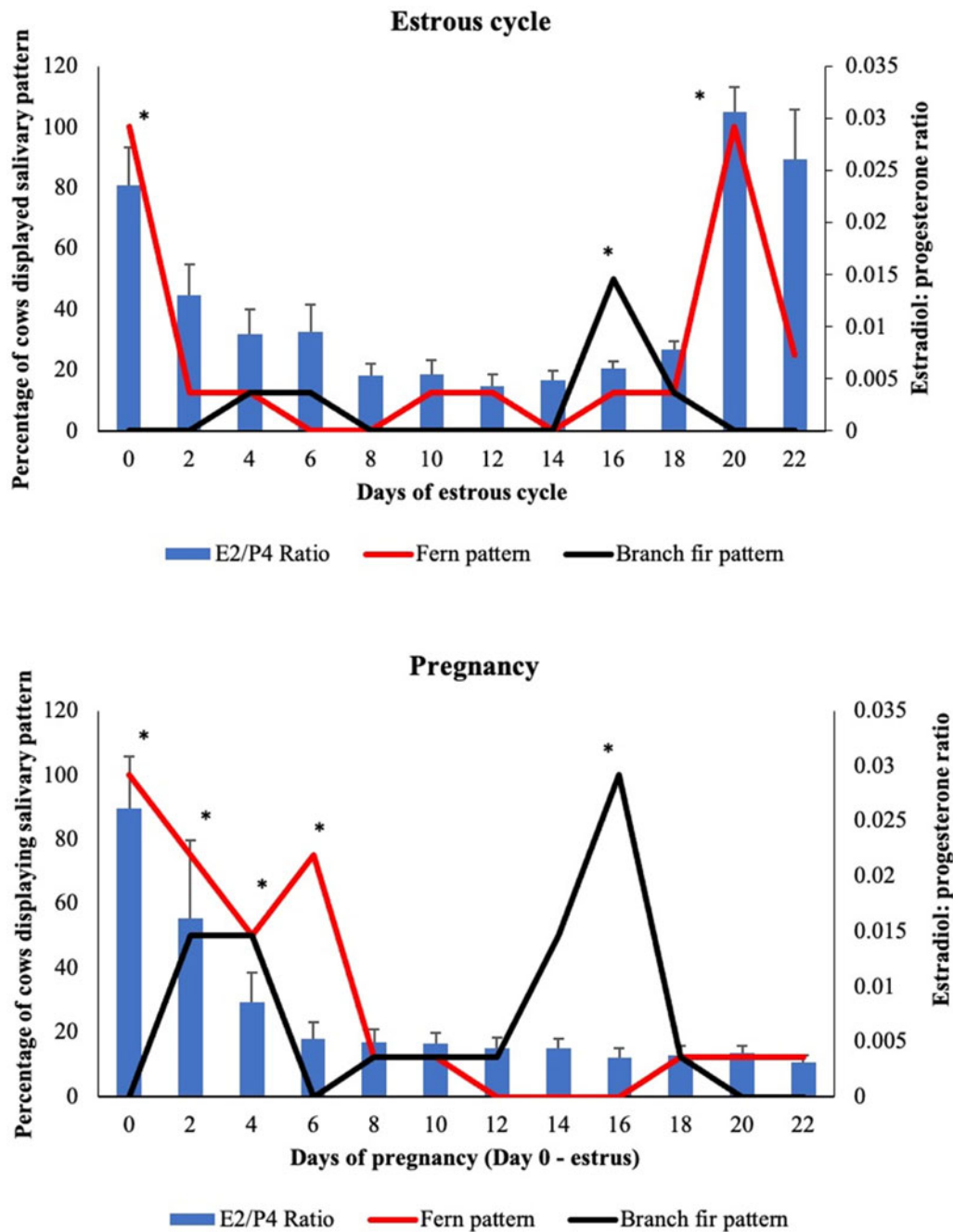


Fig. 2. The relationship between salivary crystallization pattern (fern-like and branch-fir pattern) and estradiol:progesterone ratio during (top panel) estrous cycle and (bottom panel) early pregnancy in zebu cows. *Significantly different from random occurrence ($P < 0.001$).

appearance of salivary crystallization pattern, was that all the pregnant cows showed branch- and fir-like pattern on day 16 of pregnancy. Only one previous study reported the salivary crystallization pattern in relation to pregnancy diagnosis in Holstein cows (Skalova *et al.*, 2013). These researchers found significant differences in crystalline patterns between pregnant or non-pregnant animals from 20 to 29 d after AI. They reported that branch like salivary crystallization pattern predominated in pregnant animals, which is in agreement with our findings.

In conclusion, the findings of the present study confirms that the salivary crystallization pattern differs with the stage of estrous

cycle and pregnancy in dairy cattle. A typical fern-like salivary crystallization pattern was observed on the day of estrus. At 20 d post-insemination, all the non-pregnant cows showed this same crystallization pattern while only one of the pregnant cows did. Further, all the pregnant cows showed a combination of branch and fir-like pattern on day 16 of pregnancy.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S002202922000887>.

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