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# Use of Salivary Fern Patterns for Early Pregnancy Diagnosis in Beef Cattle

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**Key Words**
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**ABSTRACT**

We examined the presence of and changes in saliva fern patterns in beef cattle during estrus and early pregnancy in 114 cyclic cows in the postpartum period. Saliva samples were collected on the day of artificial insemination (AI) and day 21 post-AI and assessed microscopically. Saliva fern patterns were classed as: No fern, dotted fern, star-like fern, branch-like fern and fern-like fern. Pregnant cows were examined on day 21 post-AI and serum progesterone was analyzed by immunoassay. Rectal palpation on day 60 post-AI to reconfirm the pregnancies revealed that 47 cows were non-pregnant and 67 were pregnant. Serum progesterone levels $\geq$2.5 ng $mL^{-1}$ on day 21 post-AI to diagnose pregnancy showed 100% accuracy in non-pregnant cows and 85.07% accuracy in pregnant cows. The fern pattern of saliva on AI day showed only fern pattern (0% no fern). However, there were no significant differences in the salivary fern patterns of non-pregnant and pregnant cows on the day of AI ($p>0.05$). We found significant differences in the salivary fern pattern between pregnant and non-pregnant cows on day 21 post-AI ($p\leq0.05$), with non-pregnant cows showing only fern pattern (0% no fern), where as both no fern and fern patterns appeared in the saliva of pregnant cows. The no fern pattern observed in saliva on day 21 post-AI method confirmed pregnancy in 29.85% of pregnant cows. Early pregnancy diagnosis using salivary fern pattern maybe possible, however, its practical application is currently unfeasible.

## INTRODUCTION

Beef cows need to produce a calf every year to be economically viable. An annual pregnancy examination for every cow is an important management tool that will improve the reproductive efficiency and detect any problems early in the breeding cycle. Currently, pregnancy examination and early pregnancy diagnosis are achieved by measuring progesterone levels in blood or milk 18-24 days post-AI Lucy *et al*.[1] and Bekele *et al*.[2] and transrectal ultrasonography to detect embryos 28-35 days post-AI[2-5], however, these are expensive, time consuming and require veterinary assistance.

Several recent studies of salivary fern morphology in livestock reproductive monitoring have examined sheep[6], cattle[7-9] and buffalo[10-13]. These studies have reported several advantages of salivary fern testing, such as its high accuracy, low cost, convenience, reusable, ease of use and ability to be performed by the farmer. Fern patterns in saliva have been shown to be correlated with the estrous cycle. Ferning occurs due to the presence of sodium chloride, which forms fern-like patterns due to its crystallization in saliva in response to estrogen during ovulation and does not appear in other phases of the estrous cycle in animals. A typical salivary fern pattern is distinctive near the peak of follicular activity and around the time of ovulation when estrogen predominates[6,7,9].

Therefore, we hypothesized that ferning would differ according to hormonal changes during the estrus cycle and pregnancy, consistent with the different fern morphologies in saliva. This method may be applied to pregnancy during early pregnancy in cows. The present study aimed to confirm the presence of and changes in salivary fern patterns in beef cattle cows during the estrous cycle and early pregnancy as well as differences in salivary fern patterns between non-pregnant and pregnant cows.

## MATERIALS AND METHODS

**Animals:** The present study was carried out at a beef cattle farm at Tubkwang Research Station, Department of Animal Science, Faculty of Agriculture, Kasetsart University, Saraburi, Thailand. The breed used was Kamphaeng Saen, which is a crossbred cattle whose bloodline contains 25% native Thai cattle, 25% Brahman and 50% Charolais. The study included 114 cyclic cows aged between 4 and 12 years in the postpartum period (90 days) with a body condition score of 6.31±0.74, where 1 represents emaciated and 9 represents fat[14]. All cows were housed in a dirt lot with an indoor feeding area under good care conditions at a temperature of 29.67°C±2.45°C and temperature humidity index of 80.66±2.97. All cows grazed on Ruzigrass (*Brachiaria ruziziensis*) and Napier grass (*Pennisetum purpureum*) and had free access to water.

**Estrus detection and AI:** Estrus detection in cows was carried out intensively twice daily (06.00-07.00 am and 06.00-07.00 pm) by experienced persons. Standing estrus behavior is when a cow/heifer stands still and allows other cattle to be mounted, which a cow that stands still is in standing estrus and ready to continue artificial insemination. Cows in standing estrus underwent AI using the freeze-thawed semen of a fertility-proven bull at 12 h after standing estrus. Pregnancy was determined using rectal palpation on day 60 post-AI.

**Saliva collection and salivary fern pattern:** Saliva samples were collected from each cow on the day of AI and day 21 post-AI using a sterile cotton swab to sweep the tongue. A drop of non foamy saliva sample was then smeared on a glass slide and air-dried at room temperature. The smear samples were microscopically assessed at 100× magnification. Salivary fern patterns were classified according to an adapted system as follows: No fern (N), dotted fern (D), star-like fern (S), branch-like fern (B) and fern-like fern (F) (Fig. 1). Only one general type of salivary fern pattern was described in each sample. Salivary fern patterns were observed and photographed.

**Blood collection and progesterone quantification:** Blood samples were collected from all cows via the coccygeal vein on day 21 post-AI. Blood was collected into 10 mL tubes (Monovette, Sarstedt AG and Co, Numbrecht, Germany), which were immediately placed on a rack and stored at 25°C for at least 3 hrs to allow clotting. Serum was then harvested and stored at -20°C until used for progesterone analysis. Serum progesterone was measured using a fully automated analyzer (ARCHITECT i2000sr) using Chemiluminescent Microparticle Immunoassay (CMIA, Bria Lab, Bangkok R.I.A. Group, Bangkok, Thailand, ISO 15189:2012). Serum progesterone levels >2.5 ng $mL^{-1}$ (cut-off value) on day 21 post-AI were considered to indicate pregnancy[15].

**Statistical analysis:** All statistical analyses were performed using R version 3.6.1[16]. Differences in the salivary fern patterns between pregnant and non-pregnant cows on the day of AI and day 21 post-AI were tested by Chi-Square test. The $p \leq 0.05$ were considered significant.

**Ethics statement:** The present study was approved by Kasetsart University's Institutional Animal Care and Use Committee (approval no. ACKU63-AGR-006).

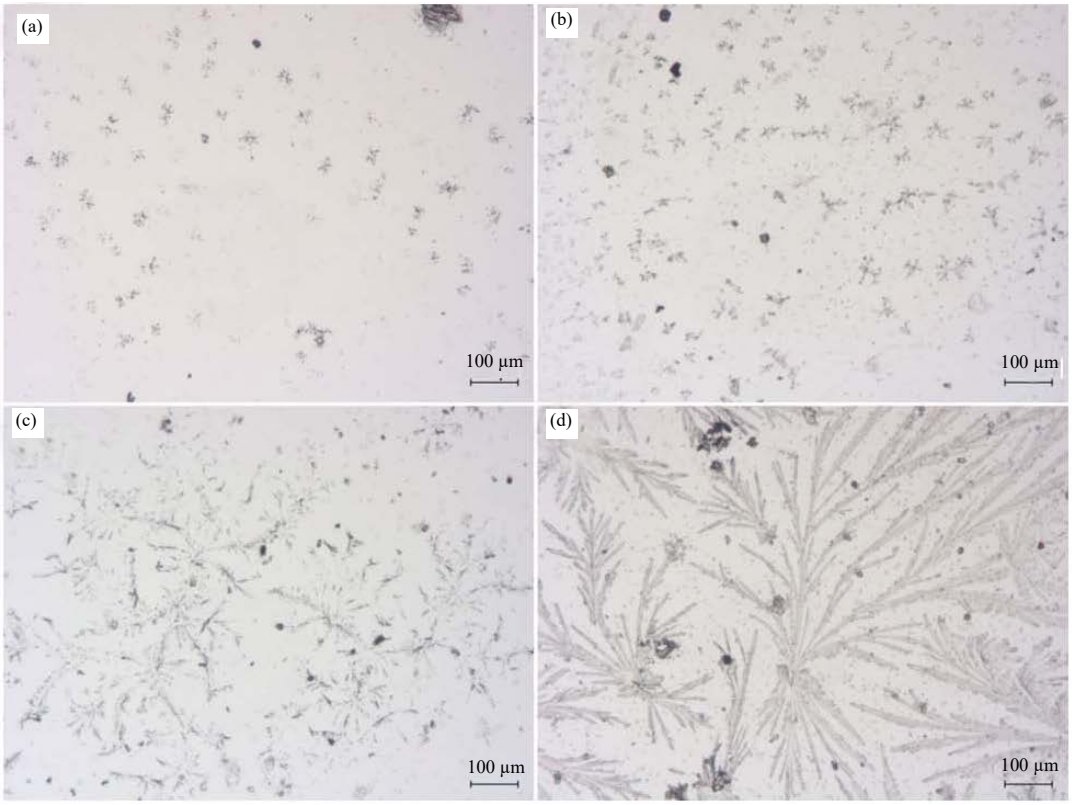


Fig. 1(a-d): Saliva morphology: (a) Dotted fern, (b) Star-like fern, (c) Branch-like fern and (d) Fern-like fern

**RESULTS**

All cows underwent a pregnancy examination on day 21 post-AI using serum progesterone analysis. A total of 47 cows were non-pregnant on day 21 post-AI, with serum progesterone levels of 0.10–1.97 ng $mL^{-1}$ (Fig. 2). Pregnancy status was reassessed by rectal palpation on day 60 post-AI and all the cows found to be non-pregnant on day 21 post-AI gave the same non-pregnant test results. A total of 67 cows were diagnosed to be pregnant on day 21 post-AI and had serum progesterone levels of 2.63-13.73 ng $mL^{-1}$ (Fig. 2). However, pregnancy reassessment on day 60 post-AI found that 57 cows remained pregnant (serum progesterone levels of 3.59-10.28 ng $mL^{-1}$ on day 21 post-AI) and 10 were non-pregnant.

Among saliva samples collected from 114 cows, the saliva morphology on the day of AI showed the following proportions of salivary fern pattern: 0% N, 14.91% D, 4.39% S, 29.82% B and 50.88% F (Fig. 4). The saliva morphology on the day of AI of non-pregnant cows showed the following proportions of salivary fern patterns: 0% N, 21.28% D, 4.26% S, 27.66% B and 46.81% F (Fig. 3). The saliva morphology of pregnant cows on the day of AI showed the following proportions of salivary fern patterns: 0% N, 10.45% D, 4.48% S, 31.34% B and 53.73% F (Fig. 3). However, there were no significant differences in the proportions of the salivary fern patterns between non-pregnant and pregnant cows ($p>0.05$).

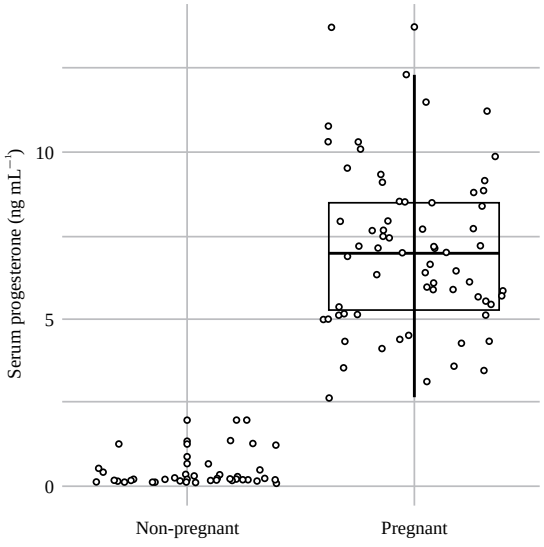


Fig. 2: Boxplot of a comparison of serum progesterone levels on day 21 post-AI between non-pregnant and pregnant cows

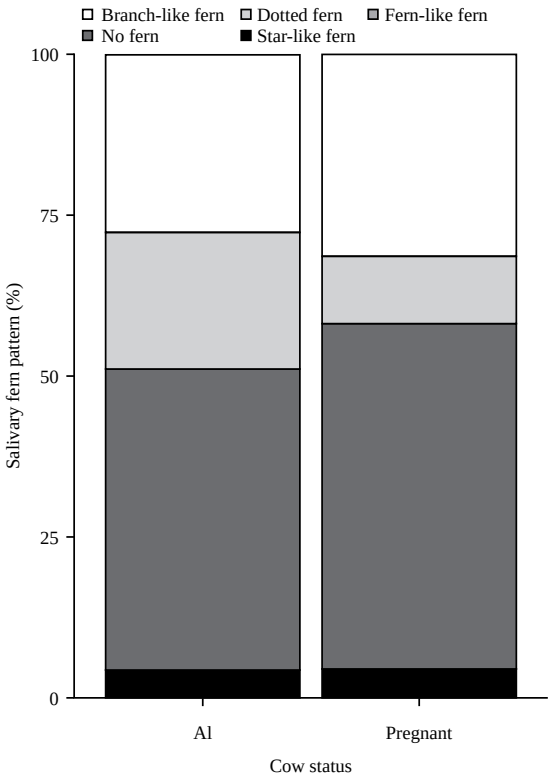


Fig. 3: Boxplot of a comparison of the proportions of the salivary fern pattern on the day of AI between non-pregnant and pregnant cows

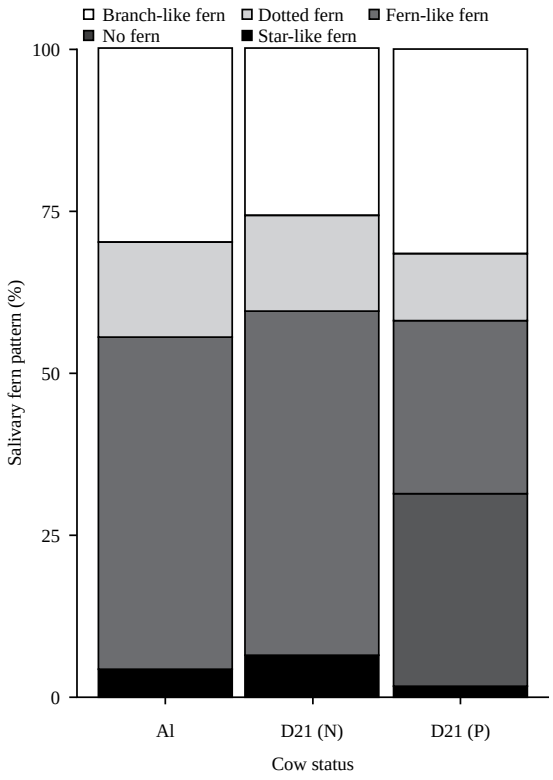


Fig. 4: Boxplot of a comparison of the proportions of the salivary fern patterns on day 21 post-AI between non-pregnant (N) and pregnant cows (P)

The saliva morphology of non-pregnant cows on day 21 post-AI showed the following proportions of salivary fern patterns: 0% N, 14.89% D, 6.38% S, 25.53% B and 53.19% F (Fig. 4). The saliva morphology of pregnant cows on day 21 post-AI showed the following proportions of salivary fern patterns: 29.85% N, 10.45% D, 1.49% S, 31.34% B and 26.87% F (Fig. 4). However, there was significant difference in the proportions of the salivary fern patterns between pregnant and non-pregnant cows ($p \leq 0.05$).

## DISCUSSION

Serum progesterone levels were measured in pregnant cows on day 21 post-AI using the CMIA method. Use of a serum progesterone cut-off level of $\geq 2.5$ ng $mL^{-1}$ to diagnose pregnancy showed an accuracy of 100% in non-pregnant cows and 85.07% pregnant cows when pregnancy was reconfirmed by rectal palpation on day 60 post-AI. Similarly, several studies have reported the use of pregnancy test methods using progesterone in milk and blood at 19-24 days after AI and showed 100% accuracy for non-pregnant cows and 75-85% accuracy for pregnant cows[15,17,18]. Embryonic loss was further divided into two categories and classified as early embryonic mortality (fertilization-day 27) and late embryonic mortality (days 28-42). The majority of embryonic mortality was early embryonic mortality, with rates ranging from 20-44% reported in beef cattle[19]. Late embryonic mortality occurs in 3-14% of beef cows and heifers[19,20]. The main factors implicated in embryonic or fetal loss are often genetic, physiological, endocrine, or environmental[21,22].

In the present study, the fern pattern of saliva showed only the fern pattern on the day of estrus. The phenomenon of ferning is affected by estrogen and progesterone levels. During the follicular phase of the estrus cycle, saliva estrogen levels increase with increases in blood estrogen levels[11,23]. Interactions between estrogen and electrolytes (specifically NaCl) result in a ferning appearance in saliva, which is a crystal configuration resembling fern leaves[6,7,9,11]. Among these salivary fern patterns, a typical clear fern-like pattern was observed at days 0-1 duration the estrus cycle, followed by ovulation approximately within 24-72 hrs in Umblachery cattle[9].

We found significant differences in the proportions of the salivary fern patterns in pregnant and non-pregnant cows on day 21 post-AI. The salivary

fern pattern of non-pregnant cows on day 21 post-AI showed only the fern pattern (0% no fern), where as both no fern and fern patterns appeared in the saliva of pregnant cows. A similar trend was reported by Skalova *et al*.[7], who found predominantly no fern or branch-like fern patterns in the saliva of pregnant cows, whereas the fern-like fern pattern was found relatively more frequently in the saliva of non-pregnant cows between days 20 and 29 post-AI. Kandiel *et al*.[12] found that the peak incidence of the saliva ferning patterns showed mainly branch-like fern pattern on days 20-25 and 28 post-breeding in pregnant buffaloes and fern-like fern pattern on days 15-28 post-breeding in non-pregnant buffaloes.

There were no significant differences in the salivary fern patterns between the day of AI and day 21 post-AI in non-pregnant cows, whereas there were significant differences in pregnant cows. This finding corresponds with the return to estrus in non-pregnant cows approximately 21 days after AI and hormonal changes[24,25].

## CONCLUSION

Our findings indicate that pregnancy diagnosis using salivary fern patterns in beef cattle cow maybe possible by observing the salivary fern patterns in saliva. The appearance of no fern in the saliva indicates that the cow is pregnant. The appearance of fern pattern in the saliva indicates that the cows are not pregnant, as well as observing estrus symptoms around 21 days post-AI, which is the time at which estrus returns when AI is unsuccessful.

## ACKNOWLEDGMENTS

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