



Utilizing Vaginal Cytology and Gelatin Zymography for Pregnancy Diagnosis in Pigs

ADSADAWUT SANANNAM¹, WORAWATT HANTHONGKUL², YU-JING LIAO³, KUNLAYAPHAT WUTHIJAREE⁴, PATTARAPORN TATSAPONG⁴, AMORN RAT WANANGKARN^{4,5}, ANURAK KHIEOKHAJONKHET^{4,5}, NIRAN AEKSIRI^{4,5}, SUREEPORN SAENGWONG⁶, WILASINEE INYAWILERT^{4,5*}

¹Department of Animal Science, Faculty of Sciences and Agricultural Technology, Rajamangala University of Technology Lanna, Phitsanulok, Thailand; ²Phitsanulok artificial insemination and biotechnology research center, Phitsanulok, Thailand; ³Genetics and Physiology Division, Taiwan Livestock Research Institute, Ministry of Agriculture, Tainan, Taiwan; ⁴Department of Agricultural Science, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand; ⁵The Center for Agricultural Biotechnology, Naresuan University, Phitsanulok, Thailand; ⁶Division of Animal Science, School of Agriculture and Natural Resources, University of Phayao, Phayao, Thailand.

Abstract | Timely identification of non-pregnant sows and gilts in the early stages can greatly enhance reproductive efficiency and financial success in pig farming. Vaginal cytology is a reliable and direct technique used to identify the estrus phase and can distinguish animals that are returning to the estrus stage. Furthermore, matrix metalloproteinases (MMPs) are involved in altering the extracellular matrix (ECM) throughout pregnancy and parturition. The aim of this study was to examine changes in the characteristics of vaginal cells and alterations in the expression of matrix metalloproteinases (MMPs) using gelatin zymography in pregnant pigs. Eight sexually mature individuals were identified as being in early pregnancy using the vaginal smear technique within 18 to 26 days following artificial insemination. The findings indicated an elevated leukocyte count in pregnant pigs between days 18 and 26. However, a small number of epithelial cells that were either nucleated or cornified were observed during the entire pregnancy period. Additionally, the MMP-2 and MMP-9 expression was analyzed using gelatin zymography in four pregnant pigs on day 60 and a non-pregnant pig on the day it returned to estrus after artificial insemination. Each sample was standardized by dividing the density of the band using Image J Software. Pregnant pigs showed significantly higher levels of MMP-9 compared to non-pregnant pigs ($p \leq 0.05$). While the level of MMP-2 expression was greater in pregnant pigs, there was no statistically significant differences in MMP-2 levels seen between pregnant and non-pregnant pigs ($p \geq 0.05$). Thus, vaginal cytology is a direct and efficient technique for detecting pregnancy in pigs. Furthermore, further investigation is necessary to evaluate MMP-9 as a potential biomarker for confirming pregnancy in pigs, with the objective of enhancing the precision of pregnancy detection in female pigs and resolving notable obstacles in the swine breeding industry.

Keywords | Vaginal cytology, Gelatin zymography, Pregnancy, Pig

Received | July 17, 2024; **Accepted** | August 04, 2024; **Published** | August 30, 2024

***Correspondence** | Wilasinee Inyawilert, Department of Agricultural Science, Faculty of Agriculture Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand; **Email:** wilasineei@nu.ac.th

Citation | Sanannam A, Hanthongkul W, Liao YJ, Wuthijaree K, Tatsapong P, Wanangkarn A, Khieokhajonkheth A, Aeksiri N, Saengwong S, Inyawilert W (2024). Utilizing vaginal cytology and gelatin zymography for pregnancy diagnosis in pigs Adv. Anim. Vet. Sci. 12(10): 2015-2021.

DOI | <https://dx.doi.org/10.17582/journal.aavs/2024/12.10.2015.2021>

ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331



Copyright © 2024 by the authors. Licensee ResearchersLinks Ltd, England, UK.

This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Early detection of nonpregnant sows and gilts can significantly improve reproductive efficiency and financial profitability in pig farming. This can be achieved by reducing the number of unproductive days per sow each year (Williams *et al.*, 2008). There are multiple methods for detecting estrus. The application of a boar for heat detection demonstrates an exceptional accuracy rate of 75–96% (Lee *et al.*, 2019). Nevertheless, the precision of heat perception in female pigs can vary substantially among different groups of animals, necessitating caution when using this technique for detecting open sows that exhibit signs of estrus vary from 17 to 25 days depending on breed and individual differences (Almond and Dial, 1986; Glossop and Foulkes, 1988; Sharifuzzaman *et al.*, 2024). Alternatives include evaluating urine oestrogens, oestrone sulphate assays, and ultrasonography techniques (Kousenidis *et al.*, 2022). Currently, B-ultrasound is widely used for early pregnancy identification but is not effective in identifying pregnancy during the first estrus cycle (21 days) after artificial insemination, resulting in a rise of unproductive days (Ren *et al.*, 2024).

Vaginal cytology is a dependable and simple method used to determine the estrus stage and can identify animals that do or do not return to estrus. The vaginal discharge consists of four types of cells: parabasal cells, intermediate cells, superficial epithelial cells, and leukocytes. These cells display different patterns throughout the estrous cycle (Lorenzen *et al.*, 2016; Rodgers *et al.*, 1993). Sexually mature minipigs showed a dominance of parabasal cells during diestrus, whereas during estrus, neutrophils and other leukocytes increased, becoming the predominant cell types (Lorenzen *et al.*, 2016). In a study conducted on Yucatan minipigs, it was shown that the vaginal leukocytes were consistently higher than epithelial cells throughout the cycle, except during estrus.

which were primarily composed of superficial epithelial cells. Superficial and intermediate cells increase throughout proestrus (Lorenzen *et al.*, 2016). In goats, superficial cells were more commonly detected and were found to be linked to the proestrus, estrus, and early metestrus phases of the reproductive cycle. Intermediate and parabasal cells were observed during the diestrus phase (Ola *et al.*, 2006). Moreover, vaginal cytology has been used to detect pregnancy in various animals such as deer mice, cows, sheep, and goats (Ola *et al.*, 2006; Paull and Fairbrother, 1985; Sharma and Sharma, 2016). During goat pregnancy, a vaginal smear usually displays a small number of intermediate cells, minimal amount of red blood cells, and a substantial quantity of polymorphonuclear cells (Sharma and Sharma, 2016). A previous study (Ali *et al.*, 2020) examined the early stages of pregnancy and detailed the corresponding characteristics of pregnancy from day 22 to day 40 using vaginal cytology. The results indicated that the mean percentage of interme-

diate cells (81.12%) was substantially greater than that of the superficial (9.41%), keratinized (7.10%), and neutrophil (2.61%) cells ($p < 0.05$). Based on these findings, it is evident that intermediate and parabasal cells were more prevalent throughout the diestrus phase and early stages of pregnancy.

We hypothesized that changes in vaginal discharge during the estrus cycle or pregnancy could be associated with estrogen levels. Applying synthetic E2 topically can help decrease alterations in the vaginal environment, indicating that E2 signaling in the vagina is involved in maintaining immune response and vaginal equilibrium. Research has demonstrated the vital role of epithelial estrogen receptor α (ESR1) in immunological suppression. Without epithelial ESR1, the inhibition of vaginal leukocytes can be compromised, leading to increased MMP activity. MMPs are enzymes that break down proteins in the cellular matrix, leading to changes in the extracellular matrix (ECM) and cell detachment. (Li *et al.*, 2018). This study aimed to examine the changes in vaginal cytology characteristics and MMPs expression using gelatin zymography in pregnant pigs.

MATERIALS AND METHODS

ANIMAL

All pig experiments were approved in advance by the Institutional Animal Use and Care Committee (IACUC) of Rajamangala University of Technology Lanna, Thailand (RMUTL-IACUC 021/2023). Eight sexually mature, approximately 360 days old, were used to determine pregnancy following artificial insemination. The pigs were kept in individual compartments within the animal facility, with each compartment separated by vertical bars. In addition, the pigs were reared under controlled conditions, ensuring they had unrestricted access to water and were fed a standardized diet tailored to meet their nutritional needs. The animals were provided with food on two occasions daily, specifically at 8:00 a.m. and 3:00 p.m. Each pig was given unrestricted access to water and was fed around 2.5 to 3.0 kilogram of feed each day. Estrus identification was performed on a daily basis using visual assessments and the back-pressure test to ascertain the presence of the standing reaction, vulvar reddening, and swelling. In addition, boar introduction was conducted daily to encourage the animals.

VAGINAL CYTOLOGY

Artificial insemination was performed on eight sexually mature individuals, approximately 360 days old, and early pregnancy was confirmed using the vaginal smear technique 18 to 26 days following the procedure. A sterile cotton-tipped swab moistened with normal saline was gently inserted into the gilt's vagina to a depth of approximately 4 inches, collecting cells. The swab was gently rotated and moved within the vagina to ensure cell collection. The cells were then transferred to a preheated dry glass slide by mov-

ing the swab across the slide's surface. Subsequently, the slides were air-dried at room temperature for 2 minutes and stained with Wright-Giemsa stain from Sigma-Aldrich for an additional 2 minutes. The slides were washed with water, air-dried, and immediately examined under a light microscopy at 100 x magnification. Vaginal epithelial cells, including parabasal, intermediate, superficial, and leukocyte cells, were identified and categorized in both pregnant and non-pregnant gilts.

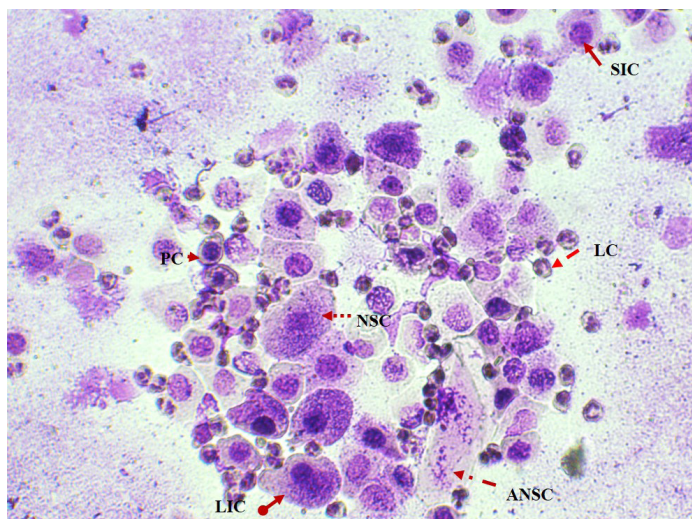


Figure 1: The vaginal cytology of female pigs exhibits various types of epithelial cells, including parabasal cells (PC), small intermediate cells (SIC), large intermediate cells (LIC), nucleated superficial cells (NSC), anucleated superficial cells (ANSC), and leukocytes (LC).

GELATIN ZYMOGRAPHY

For gelatin zymography, two milliliters of blood were collected from the marginal ear veins of four pregnant pigs on day 60 after confirmation by ultrasonography as well as four non-pregnant pigs on the day it returned to estrus after artificial insemination. The samples were allowed to coagulate at room temperature and then centrifuged at 2,500 g for 15 minutes to separate the serum. The serum was carefully collected and the protein concentration of the liquid above the sediment was measured. Samples not immediately analyzed were stored at -20 °C until analysis. A total of 25 grams of protein were mixed with a sample buffer at pH 6.8, containing 62.5 mM Tris-HCl, 25% glycerol, 4% SDS, and 0.01% bromophenol blue (Bio-Rad) in a 1:1 ratio. The resulting mixture was then subjected to electrophoresis on a 7.5% polyacrylamide gel with sodium dodecyl sulfate (SDS) and 1mg/ml gelatin in the resolving gel (Minigel, Bio-Rad, California, USA; Sigma-Aldrich, Saint Louis, MO, USA). Following electrophoresis, the gel was washed with 2.5% TritonX100 at room temperature (25°C) for 30 minutes to remove SDS. Subsequently, the gels were exposed overnight at 37°C to a solution containing 50 mM Tris-base solution (Bio-Rad, California, USA) with pH 7.4, 200 mM NaCl, 0.02% Brij-35, and 5 mM

CaCl₂, resulting in the development of zymolytic bands. The next day, the gels were stained with a solution containing 0.5% Coomassie Blue R-250 (Sigma-Aldrich) in a mixture of 40% methanol, 10% glacial acetic acid, and 50% distilled water for 30 minutes. The samples were subsequently rinsed with a solution containing 30% methanol, 7.5% glacial acetic acid, and 62.5% distilled water for 30 minutes. Subsequently, they were stored in distilled water. The gelatinase activity could be visualized as bars on a blue background, specifically matrix metalloproteinase-2 and -9. The obtained image was processed using ImageJ software version 1.80 from the National Institute of Mental Health in Bethesda, Maryland, USA (Schneider *et al.*, 2012). The area of the bands was measured as a representation of gelatinase activity.

STATISTIC

T-tests were used to determine the statistical significance of the observed changes in gelatinase activity (MMP-2 and MMP-9) between pregnant and non-pregnant pigs. The intergroup variability, as indicated by the t-tests, confirms the accuracy of the observed distinctions. The statistical study was performed using R version 4.1.2, developed by the R Core Team in 2021.

RESULTS AND DISCUSSIONS

VAGINAL CYTOLOGY FOR PREGNANCY DIAGNOSIS

The sexual maturity and pregnancy status of female pigs were assessed by examining vaginal smears to identify several types of cells, including nucleated superficial epithelial cells, anucleated superficial epithelial cells, small intermediate cells, large intermediate cells, and leukocytes (Figure 1). Pregnancy confirmation was achieved by monitoring estrus activity within a period of 18 to 26 days after artificial insemination. The results suggested a higher number of leukocytes in the pregnant pig during days 18 to 26. Nevertheless, a limited number of nucleated and cornified epithelial cells were detected throughout the entire duration of pregnancy, as depicted in Figure 2 A-I. In contrast, results from nonpregnant pigs showed that superficial epithelial cells were the most common type during the return to estrus stage (Figure 3 A-C). After the estrus stage, there was a total decrease in nucleated and anucleated superficial epithelial cells, with only a small amount of parabasal, intermediate, and leukocyte cells found on days 21-24 (Figure 3 D-G). In addition, there was a greater prevalence of Leukocyte cells on days 25-26, as shown in (Figure 3 H and I). Furthermore, the pregnancy rate was determined using techniques such as vaginal smear, estrus determination. The pregnancy status of each female pig was assessed by examining vaginal smears 18 to 26 days following artificial insemination (AI). By employing the vaginal smear technique, we have verified that out of the total of eight pigs, four were found to be pregnant while the remaining

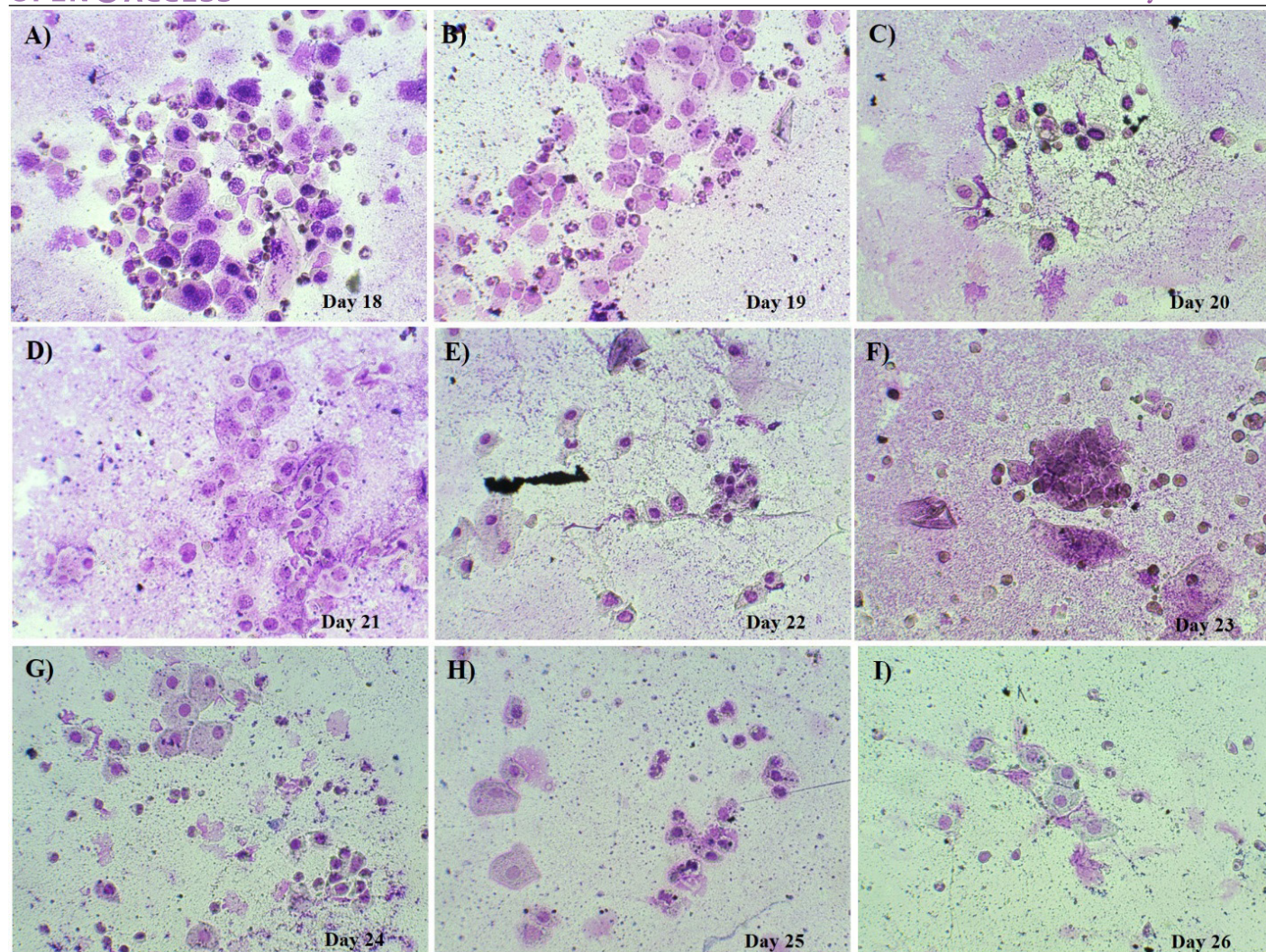


Figure 2: Representative vaginal smear images from pregnant pigs during days 18 to 26 after insemination, showing a higher number of leukocytes and a limited number of nucleated and cornified epithelial cells.

four were not pregnant. The vaginal smear approach demonstrated a pregnancy detection accuracy of approximately 100% (Table 1).

Table 1: The comparison of pregnancy diagnosis methods between vaginal cytology and estrus detection.

Method of pregnancy diagnosis	No. of animal	Pregnancy	Non-Pregnancy
Vaginal cytology	8	4	4
Estrus detection	8	4	4
% Accuracy vaginal cytology	8	100	100
% Accuracy Estrus detection	8	100	100

ANALYSIS OF MMP-2 AND MMP-9 EXPRESSION IN PREGNANT AND NON-PREGNANT PIGS USING GELATIN ZYMOGRAPHY

Blood samples were obtained from pregnant pigs on the 60th day of pregnancy and from a nonpregnant pigs on the day of estrus following artificial insemination. The study employed gelatin zymography and densitometric analysis using ImageJ software to determine the expression levels of

the typical form of MMP-2 and MMP-9. Figure 3 illustrates the progressive alterations in the average band intensities corresponding to the 92 kDa (MMP-9) and 72 kDa (MMP-2) proteins. The zymogram analysis of both pregnant and non-pregnant pig serum revealed the presence of MMP-9 and MMP-2. Pregnant pigs exhibited markedly increased levels of MMP-9 compared to non-pregnant pigs ($p \leq 0.05$). Although the level of MMP-2 expression was higher in pregnant pigs, there was no statistically significant difference in MMP-2 levels between pregnant and non-pregnant pigs ($p \geq 0.05$) (Figure 4A and C).

Efficiently operated pig farms should expect a farrowing rate of 80%. When a pregnancy test is performed three weeks after artificial insemination and proper estrus detection is implemented, only a few (5-10%) of the animals that do not show signs of estrus will be determined to be not pregnant. This indicates that the costs related to a pregnancy test are influenced by a relatively small group of animals that require testing. In order for the test to be profitable, the method should have a specificity close to 100% (Taverne *et al.*, 1985).

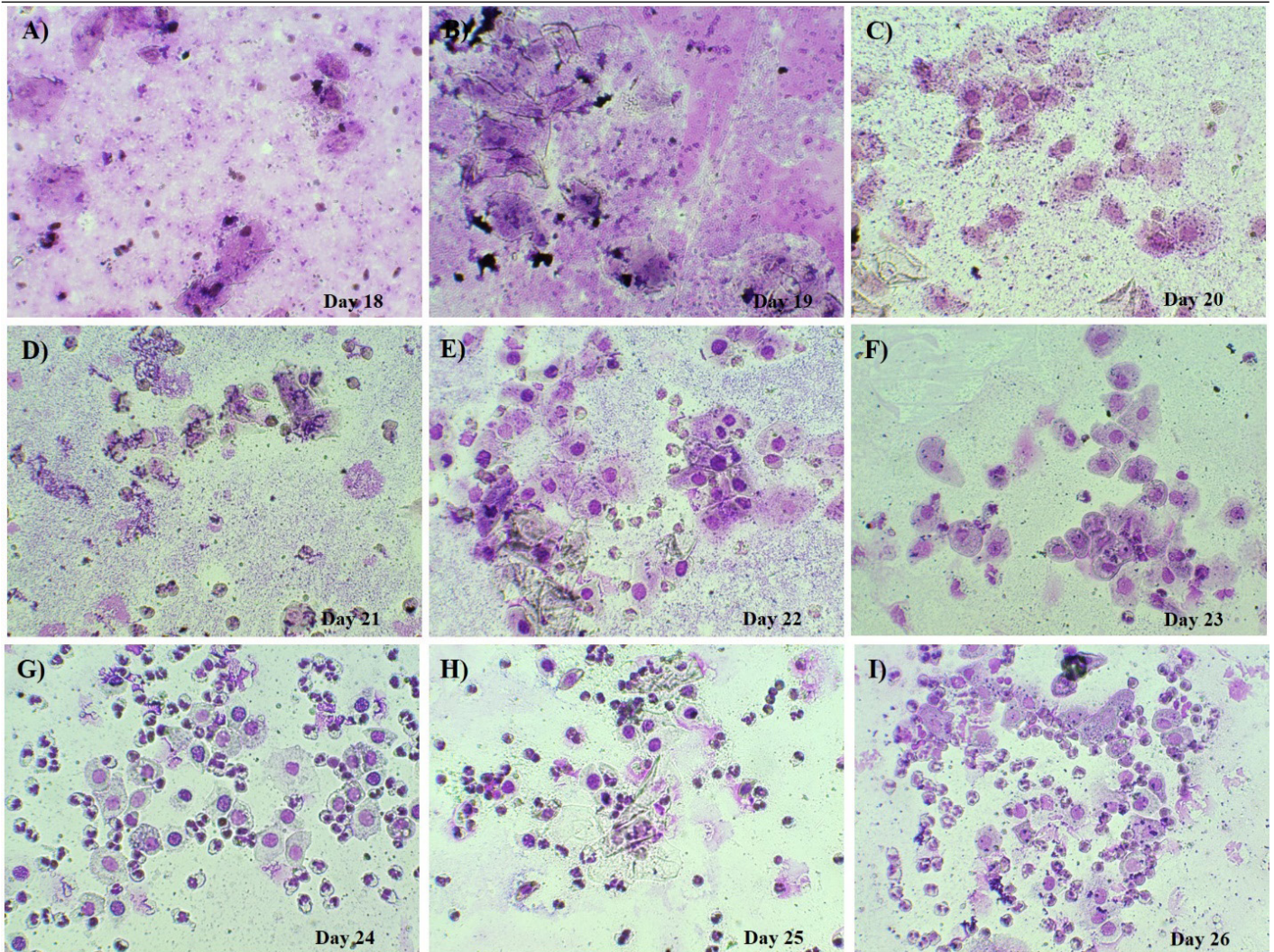


Figure 3: Representative vaginal smear images from non-pregnant pigs during the return estrus stage and subsequent days, illustrating the prevalence of different cell types at various time points.

Various techniques have been utilized to detect pregnancy in pigs, including Doppler, A-mode ultrasound, B-mode ultrasound, or real-time ultrasonography (RTU). Doppler and A-mode ultrasound are cost-effective and user-friendly, but they have a higher rate of incorrect positive and negative diagnoses. Additionally, they cannot be utilized until 30 to 35 days after conception (Botero *et al.*, 1986; Inaba *et al.*, 1983; Kauffold *et al.*, 1997; Williams *et al.*, 2008). In contrast, B-mode RTU enables pregnancy detection at 21 days after mating with greater accuracy, precision, and an efficiency rate of nearly 95% (Flowers *et al.*, 2000; Williams *et al.*, 2008).

Nevertheless, vaginal cytology served as a straightforward and cost-effective technique for early detection of pregnancy in pigs. During this experiment, the sexual maturity of eight female pigs was evaluated by analyzing vaginal smears to detect various cell types and confirming pregnancy by observing estrus activity throughout a period of 18 to 26 days following artificial insemination. The findings indicated an elevated leukocyte count in the pregnant pigs between days 18 and 26. However, a small number of nu-

cleated and cornified epithelial cells were observed during the entire pregnancy, as shown in Figure 2 A-I. Conversely, the cytological analysis of vaginal smears obtained from non-pregnant pigs revealed that superficial epithelial cells constituted the predominant cell type throughout the return to estrus stage (Figure 3 A-C). Following the estrus stage, there was a complete reduction in the number of nucleated and cornified epithelial cells, with only a minimal presence of parabasal, intermediate, and leukocyte cells observed on days 21-24 (Figure 3 D-G). Furthermore, there was a higher occurrence of Leukocyte cells on days 25-26, as depicted in (Figure 3 H and I).

The findings from this study exhibit congruence with the previously established diagnostic criteria for determining the estrus stage through vaginal cytological examination in miniature swine. A previous study found that the number of superficial and intermediate epithelial cells was substantially higher during estrus compared to proestrus or diestrus. The number of epithelial cells was shown to be lower during diestrus compared to estrus, and there was also a tendency for it to be lower during diestrus compared

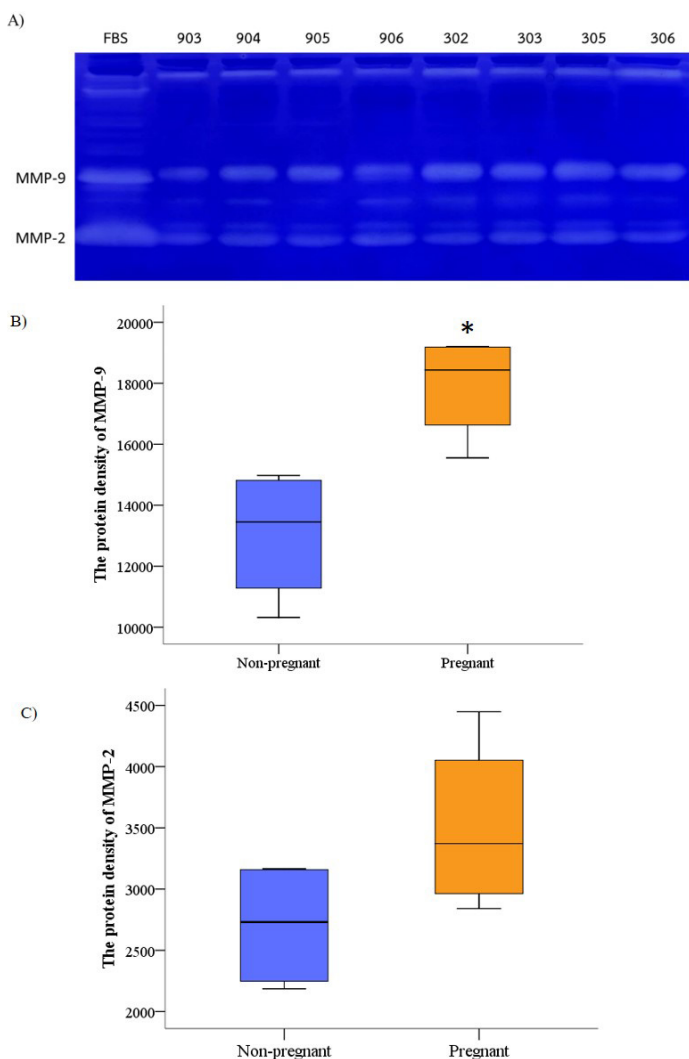


Figure 4: Gelatin zymography to determine the difference between non-pregnant and pregnant pigs at 60 days post artificial insemination. A) Gelatin zymography gel B) MMP-9 protein concentration C) MMP-2 protein level. Pigs 903, 904, 905, and 906 are not pregnant. Pigs with identification numbers 302, 303, 305, and 306 are pregnant.

to proestrus (Rodgers *et al.*, 1993). Hence, the regulation of vaginal cell changes is controlled by the sex steroid hormones estrogen (E2) and progesterone (P4) (Li *et al.*, 2018). In addition, the presence of leukocyte in mice is associated with the levels of E2 and P4 hormones in the bloodstream, and this is used to determine the stage of the estrus cycle (Cora *et al.*, 2015; Li *et al.*, 2018). The majority of cells found in vaginal smears from mice in the estrus stage are cornified. Mice in this stage are sexually responsive and open to copulation (Cora *et al.*, 2015; Li *et al.*, 2018). At estrus, the concentration of P4 rises but gradually declines towards the end of the cycle. If there is no mating, the cycle will advance to the metestrus stage (Hubscher *et al.*, 2005; Li *et al.*, 2018). At metestrus, a substantial number of leukocytes will migrate into the vaginal canal and remove the cornified cell debris. At this juncture, the levels of circulating E2 return to their typical baseline values. At

metestrus, a substantial number of leukocytes will migrate into the vaginal canal and remove the cornified cell debris. Currently, the levels of circulating E2 return to their typical baseline values. Diestrus is a phase of reproductive inactivity characterized by a reduction in the diversity of cell types observed in vaginal smears of animals. In the later phase of diestrus, there is a rise in E2 levels, which causes the cycle to advance into proestrus. Proestrus is the stage of the reproductive cycle characterized by the peak levels of E2 and luteinizing hormone, which initiate the start of the cycle. The predominant cell types observed in vaginal smears obtained from animals in proestrus are nucleated epithelial cells (Cora *et al.*, 2015; Hubscher *et al.*, 2005; Li *et al.*, 2018). Nevertheless, our study is limited by the sample size, indicating that to enhance the accuracy of the results, it is necessary to increase the sample size in future studies. Moreover, (Li *et al.*, 2018) reported that ESR1 in epithelial cells is crucial for immunological suppression. A deficiency of ESR1 expression in epithelial cells would impair the ability to suppress vaginal leukocyte activity, consequently leading to elevated levels of MMP activities. Previous research has demonstrated the presence of MMP-2 and MMP-9 enzymes in the vaginal tissues of mice during the metestrus and diestrus phases, which were affected by a reduction in ESR1 expression. Our analysis of MMP-9 expression levels in pregnant and non-pregnant pigs, utilizing gelatin zymography technique, revealed a significant rise in MMP-9 levels in pregnant pigs compared to their non-pregnant counterparts ($p \leq 0.05$). There was no substantial difference in MMP-2 levels between pregnant and non-pregnant pigs.

CONCLUSIONS AND RECOMMENDATIONS

Vaginal cytology represents a straightforward and efficient technique for detecting pregnancy in pigs. Moreover, an inquiry is required to assess MMP-9 as a viable biomarker for verifying pregnancy in pigs, aiming to improve the accuracy of pregnancy detection in female pigs and overcome significant challenges in the swine breeding sector.

ACKNOWLEDGMENTS

The authors would like to acknowledge the department of Animal Science, Faculty of Sciences and Agricultural Technology, Rajamangala University of Technology Lanna, Phitsanulok, Thailand, as well as the Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok, Thailand, for their invaluable support in providing the pig farm and necessary assistance to successfully conduct this research.

W Inyawilert and A Sanannam designed the experiment, A Sanannam, W Inyawilert, and V Hanthongkul performed the experiment, K Wuthijaree, S Saengwong, A Sanannam, and W Inyawilert analyzed the data, W Inyawilert and A Sanannam drafted the manuscript, W Inyawilert, A Sanannam, N Aeksiri, A Wanangkarn, P Tatsapong, A Khieokhajonkhet, and Y-J Liao revised and edited the manuscript.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

- Ali MA, Islam MF, Rahman SML, Zohara BF (2020). Pregnancy diagnosis in goat by using vaginal cytology and trans-abdominal ultrasonography. *J. Anim. Reprod. Biotechnol.*, 35: 338-346. <https://doi.org/10.12750/JARB.35.4.338>
- Almond GW, Dial GD (1986). Pregnancy diagnosis in swine: a comparison of the accuracies of mechanical and endocrine tests with return to estrus. *J. Am. Vet. Med. Assoc.*, 189: 1567-1571.
- Botero O, Martinat-Botté F, Bariteau F (1986). Use of ultrasound scanning in swine for detection of pregnancy and some pathological conditions. *Theriogenology*, 26: 267-278. [https://doi.org/10.1016/0093-691X\(86\)90146-9](https://doi.org/10.1016/0093-691X(86)90146-9)
- Cora MC, Kooistra L, Travlos G (2015). Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicol. Pathol.* 43: 776-793. <https://doi.org/10.1177/0192623315570339>
- Flowers WL, Armstrong JD, White SL, Woodard TO, Almond GW (2000). Real-time ultrasonography and pregnancy diagnosis in swine. *J. Anim. Sci.*, 77: 1-9. <https://doi.org/10.2527/jas2000.77E-Suppl1>
- Glossop CE, Foulkes JA (1988). Occurrence of two phases of return to oestrus in sows on commercial units. *Vet. Rec.*, 122: 163-164. <https://doi.org/10.1136/vr.122.7.163>
- Hubscher CH, Brooks DL, Johnson JR (2005). A quantitative method for assessing stages of the rat estrous cycle. *Biotech. Histochem.*, 80: 79-87. <https://doi.org/10.1080/10520290500138422>
- Inaba T, Nakazima Y, Matsui N, Imori T (1983). Early pregnancy diagnosis in sows by ultrasonic linear electronic scanning. *Theriogenology*, 20: 97-101. [https://doi.org/10.1016/0093-691X\(83\)90028-6](https://doi.org/10.1016/0093-691X(83)90028-6)
- Kauffold J, Richter A, Sobiraj A (1997). Results and experiences of a two-year investigation in the use of sonographic pregnancy control in sows at different stages of gestation. *Tierarztl. Prax. Ausg. G Grosstiere Nutztiere*, 25: 429-437.
- Kousenidis K, Kirtsanis G, Karageorgiou E, Tsiokos D (2022). Evaluation of a Numerical Real-Time Ultrasound Imaging Model for the Prediction of Litter Size in Pregnant Sows with Machine Learning. *Animals*, 12: 1948. <https://doi.org/10.3390/ani12151948>

- Lee JH, Lee DH, Yun W, Oh HJ, An JS, Kim YG, Kim GM, Cho JH (2019). Quantifiable and feasible estrus detection using the ultrasonic sensor array and digital infrared thermography. *J. Anim. Sci. Technol.*, 61: 163-169. <https://doi.org/10.5187/jast.2019.61.3.163>
- Li S, Herrera GG, Tam KK, Lizarraga JS, Beedle MT, Winuthayanon W (2018). Estrogen Action in the Epithelial Cells of the Mouse Vagina Regulates Neutrophil Infiltration and Vaginal Tissue Integrity. *Sci. Rep.* 8: 11247. <https://doi.org/10.1038/s41598-018-29423-5>
- Lorenzen E, Agerholm JS, Grossi AB, Bojesen AM, Skytte C, Erneholm K, Follmann F, Jungersen G (2016). Characterization of cytological changes IgA IgG and IL-8 levels and pH value in the vagina of prepubertal and sexually mature Ellegaard Göttingen minipigs during an estrous cycle. *Dev. Comp. Immunol.*, 59: 57-62. <https://doi.org/10.1016/j.dci.2016.01.006>
- Ola SI, Sanni WA, Egbunike G (2006). Exfoliative vaginal cytology during the oestrous cycle of West African dwarf goats. *Reprod. Nutr. Dev.*, 46: 87-95. <https://doi.org/10.1051/rnd:2005067>
- Paull JA, Fairbrother A (1985). Pregnancy diagnosis by vaginal lavage in deer mice *Peromyscus maniculatus*. *J. Exp. Zool.*, 233: 143-149. <https://doi.org/10.1002/jez.1402330121>
- Ren Y, Zhang Q, He F, Qi M, Fu B, Zhang H, Huang T (2024). Metabolomics reveals early pregnancy biomarkers in sows: a non-invasive diagnostic approach. *Front. Vet. Sci.*, 11: 1396492. <https://doi.org/10.3389/fvets.2024.1396492>
- Rodgers JB, Sherwood LC, Fink BF, Sadove RC (1993). Estrus detection by using vaginal cytologic examination in miniature swine. *Lab. Anim. Sci.*, 43: 597-602.
- Schneider CA, Rasband WS, Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9: 671-675. <https://doi.org/10.1038/nmeth.2089>
- Sharifuzzaman M, Mun HS, Ampode KMB, Laguna EB, Park HR, Kim YH, Hasan MK, Yang CJ (2024). Technological Tools and Artificial Intelligence in Estrus Detection of Sows—A Comprehensive Review. *Animals*, 14: 471. <https://doi.org/10.3390/ani14030471>
- Sharma M, Sharma N (2016). Vaginal Cytology: An Historical Perspective on its Diagnostic Use. *Adv. Anim. Vet. Sci.*, 4: 283-288. <https://doi.org/10.4103/0970-9371.190455> <https://doi.org/10.4103/0970-9371.188065>
- Taverne MA, Oving L, van Lieshout M, Willemse AH (1985). Pregnancy diagnosis in pigs: a field study comparing linear-array real-time ultrasound scanning and amplitude depth analysis. *Vet. Q.*, 7: 271-276. <https://doi.org/10.1080/01652176.1985.9693999>
- Williams SI, Piñeyro P, de la Sota RL (2008). Accuracy of pregnancy diagnosis in swine by ultrasonography. *Can. Vet. J.*, 49: 269-273.