

DETERMINATION OF EARLY PREGNANCY IN
SWAMP BUFFALOES (*BUBALUS BUBALIS*) USING VAGINAL CYTOLOGY

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ABSTRACT

The accuracy of pregnancy diagnosis is important for increasing reproductive efficiency and decreasing interbreeding intervals of swamp buffaloes. Hence, the aim of this study was to investigate the early pregnancy diagnosis in swamp buffaloes by the vaginal cytology. Thirty female buffaloes at 4 to 7 years of age were selected and administered Ovsynch (GnRH + PGF2 α + GnRH) and modified Ovsynch (GnRH + PGF2 α protocols). The vaginal swab was collected from each buffalo after 19 to 21 days of artificial insemination (AI), stained with Wright Giemsa, and observed under microscopy. The results showed that superficial epithelial cells were more dominant when rebreeding (non-pregnant) on 20 to 21 days after AI. In contrast, a vaginal smear during pregnancy was characterized by few superficial epithelial cells while the parabasal cells and leukocytes were more dominant in pregnant buffaloes on 19 to 21 days after AI. The results showed that eighteen buffaloes were pregnant, and twelve buffaloes were not pregnant using vaginal smear technique. The accuracy of vaginal smear technique was 85.71%. Moreover, pregnancy diagnosis was confirmed

using ultrasonography and rectal palpation on 90 days after AI. The results showed that twenty one buffaloes were pregnant, and nine buffaloes were not pregnant. Therefore, the vaginal cytology technique might be an alternative method to determine pregnancy and reduce estrus detection problems in swamp buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, pregnancy, vaginal cytology, swamp buffaloes

INTRODUCTION

The swamp buffalo (*Bubalus bubalis*) is an indigenous and durable breed mostly originating from northeastern Thailand, (Triwitayakorn *et al.*, 2006). Nowadays, the buffalo population is drastically reducing in Thailand. From 1995 to 2004, the number of buffaloes has been decreased from 3.7 to 1.49 million heads on an average decline of approximately 22% per year (Triwitayakorn *et al.*, 2006). As a consequence, conservation of swamp buffaloes and their sustainable utilization in the production system is of utmost necessity (Bunmee *et al.*, 2018). In this regard, artificial

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insemination (AI) can play an important role for animal conservation and improvements in the genetic capability of the animals. However, various reproductive disorders in buffalo, such as delayed puberty and maturity, poor estrus expression, long postpartum anestrus, distinct seasonal reproductive pattern, prolonged intercalving intervals, and low conception rates particularly when bred artificially, deeply affect their reproductive efficiency (Warriach *et al.*, 2015). Therefore, in order to improve the reproductive management in livestock, it is critical to establish a precise method for determination in the estrus and early pregnancy (Reese *et al.*, 2018).

A successful estrus detection technique and timing, especially before the onset of next estrus and rebreeding of those animals, can significantly improve profitability from animal farming. Recent efforts to overwhelm the problem of weak estrous behavior was assumed to be the cause of a decline in breeding efficiency in cattle and buffalo using many methods such as a vasectomized teaser, plasma and milk progesterone, estrus behavior, painting of tail, a pedometer, vaginal cytology, cervical mucus fern pattern test, vaginal temperature measurement, ultrasound, and rectal palpation (Singh *et al.*, 2000; Holmann *et al.*, 1987; Broes and LeBlanc, 2014; Narendran *et al.*, 1979; Bernardi *et al.*, 2016; Sakatani *et al.*, 2016; Honparkhe *et al.*, 2004). Vaginal cytology is a simple method and has been done for estrus detection in many animals including mice, cow, dog, goat and swamp buffalo (Suebkhampet and Marcou, 2019; Ayen *et al.*, 2012, Sharma and Sharma, 2016). Different types of epithelial cells presented in vaginal smear during the estrus cycle such as parabasal, intermediate, and superficial (cornified) cells. Parabasal cells are small-round cells with round nuclei and have the highest nucleocytoplasmic ratio. Intermediate cells are larger than that of parabasal cells. Superficial

intermediate cells are bigger than intermediate cells and have angular edges. Superficial cells are similar in size to superficial intermediate cells and nuclei are pyknotic and lacking (Ayen *et al.*, 2012). In mice, most nucleated epithelial cells present at proestrus stage when using vaginal cytology to investigate the stages of estrus cycle. The estrus stage is distinctively characterized by cornified squamous epithelial cells, which occur in clusters and no visible nuclei. The cell mixture with a predominance of leukocytes, a few nucleated epithelial, and/or cornified squamous epithelial cells show at metestrus stage. The diestrus stage predominantly consists of leukocytes (Caligioni, 2009a). In cattle, the cell samples are collected by a vaginal swab and stained with 10% giemsa to detect the estrus stage. The results show that the dominant proportion of superficial cells exhibit at estrus stage.

In addition, early pregnancy diagnosis is critical management and economic ramification because it can shorten the calving interval and rebreed cattle at the earliest opportunity (Balhara *et al.*, 2013). Nowadays, we apply many techniques to detect early pregnancy, such as rectal palpation, ultrasound, measurement of progesterone, and glycoprotein (PAG) tests; however, each technique has its own benefits and limitations (Reese *et al.*, 2018). Rectal palpation is confined even when performed by experience technicians, from approximately 45 days onwards (Perera *et al.*, 1980). Transrectal ultrasonography is an accurate method for pregnancy diagnosis as early as 26 day post-insemination, but the cost is quiet high and an appropriate veterinary technicians are necessary (Kastelic *et al.*, 1991). Occasionally, chemical based pregnancy test and the biological marker such PAGs can use for pregnancy diagnosis in blood and milk but it is limited in cost (Reese

et al., 2018). Recently, vaginal cytology has been conducted for estrus detection, but there are no available reports regarding various types of cells in vaginal smear for pregnancy diagnosis of swamp buffaloes. Therefore, in the present study, we attempted to detect the pregnancy of swamp buffaloes using vaginal cytology and provided a reference.

MATERIALS AND METHODS

Animals and fixed time artificial insemination

Thirty female swamp buffaloes at 4 to 7 years of age were selected and administered with a modified Ovsynch protocols. The swamp buffaloes in Ovsynch (n = 17) group were received gonadotropin releasing hormone (4 µg of GnRH analogue; Receptal, Intervet; 2.5 mL) on day 0, followed a PGF2α analogue (cloprostenol 250 µg; MSD Animal Health; 2 mL) on day 7. A second-treatment of GnRH analogue was given 48 h after PGF2α. In addition, the swamp buffaloes in modified Ovsynch (n = 13) group were administered with GnRH injection on day 0 and PGF2α on day 7, respectively. All swamp buffaloes were inseminated at two fixed time, 48 h and 72 h after PGF2α.

Determination of pregnancy using vaginal cytology

The AI was performed on the female swamp buffaloes at 4 to 7 years of age, and early pregnancy was determined using vaginal smear technique on 19 to 21 days after AI. To collect cells from the vagina, a sterile cotton tipped swab was rinsed with normal saline and was gently inserted into the vagina opening of swamp buffalo to depth about 4 to 5 inches. The swab was gently turned

and rolled in the vagina to remove cells. Cells were transferred to pre-heated dry glass slide by rolling the swab across the slide. The smeared slides were air-dried in room temperature for 2 minutes and then stained with Wright-Giemsa stain (Sigma-Aldrich, St. Louis, MO, USA) for 2 minutes. The slides were rinsed with water, air-dried and viewed immediately at 100 x magnification under light microscopy. The vaginal epithelial cells (parabasal, intermediate, superficial, and leukocyte cell) were observed according to each group to the determined pregnant and non-pregnant swamp buffalo. The parabasal cell is small epithelial cell, having a round shape and presenting high nuclear-cytoplasmic ratio. The intermediate cell has variation of shape and is 2 to 3 times bigger than parabasal cell. The superficial epithelial cell was large and flat but does not always have the pyknotic nucleus.

Ultrasonography and rectal palpation

The pregnancy and non- pregnancy results were confirmed using rectal amniotic sac palpation on 90 days after AI. Finally pregnancy was confirmed by rectal ultrasound examinations and performed using a portable transrectal B-mode ultrasound scanner on 90 days after AI. The uterine horns were scanned from their dorsal and lateral surfaces.

Statistical analysis

Data were analyzed using a statistical program (SAS Enterprise Guide 4.1, SAS Institute Inc., Cary, NC, USA). The significance of the differences was analyzed by chi-square test, and $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

A simple early pregnancy diagnosis method is required in domestic animals. It has important management, economic and also assists to improve strategies for adopting suitable measure for non-pregnant domestic animal because it can reduce shortening the calving interval or rebreed at the earliest opportunity (Reese *et al.*, 2018; Ingawale *et al.*, 2012). Several methods have been applied for pregnancy detection in buffaloes. Non-return to estrus was a simple but inaccurate method for early pregnancy detection because of poor estrous expression in buffaloes (Pawshé *et al.*, 1994; Abdulkareem *et al.*, 2011). Moreover, rectal palpation for detecting pregnant buffalo was used, but the positive predictive values of rectal palpation was 71.4% on days 42 to 44 after AI (Abdulkareem *et al.*, 2011). However, vaginal cytology was a simple and low cost method that was applied for early pregnancy diagnosis in swamp buffalo. In the present study, the swamp buffaloes were subjected to two estrus synchronization programs to observe the effects of estrus synchronization program on pregnancy rate. Thirty female swamp buffaloes were used to investigate the effects of administration of hormone to induce estrus synchronization. Two estrus synchronization protocols were compared. In the first estrus synchronization protocol, swamp buffaloes were received gonadotropin releasing hormone on day 0, followed a PGF2 α analogue on day 7. A second-treatment of GnRH analogue was given 48 h after PGF2 α . In the second protocol, swamp buffaloes were administered with GnRH injection on day 0 and PGF2 α on day 7. All swamp buffaloes were inseminated at the fixed time. Pregnancy diagnosis was performed using ultrasonography and rectal palpation on day 90 after insemination. The results

showed that the percentage of pregnancy in GnRH + PGF2 α protocol (84.61%) was higher about 25.79% than that in GnRH + PGF2 α + GnRH protocol (58.82%) (Table 1).

In addition, vaginal smear technique, rectal palpation and ultrasonography were used to determine the pregnancy rate. The pregnancy and non-pregnancy in each female swamp buffalo was determined by vaginal smear after 19 to 21 days of AI. Using vaginal smear technique, we confirmed that among thirty swamp buffaloes, eighteen buffaloes were pregnant, and twelve buffaloes were not pregnant. The accuracy of vaginal smear technique for pregnancy detection was about 85.71% (Table 2).

Several types of cells such as leukocyte, parabasal, intermediate, and superficial epithelial cells, were presented in vaginal smears. Based on our results, pregnant female swamp buffaloes after 19 to 21 days of AI showed a lot of leukocyte, parabasal and intermediate cells. Less superficial cells presented on day 19 to 21 after AI (Figure 1 A-C). In contrast, non-pregnant female swamp buffaloes presented numerous superficial epithelial cells with nuclei on day 19 after AI. However, on day 20 after AI, the superficial cells without nuclei were dominant, and the parabasal, intermediate, and leukocyte cells presented on day 21 after AI (Figure 2).

Therefore, vaginal cell patterns between pregnant and non-pregnant swamp buffaloes were discriminable. In previous study, vaginal cytology during estrus cycle was studied in many animals such as mouse, rat, canine, goat, sheep, and cattle (Byers *et al.*, 2012; Caligioni, 2009b; Cora *et al.*, 2015, Siregar *et al.*, 2016; Sitaresmi *et al.*, 2018). In mice, nucleated epithelial cells are dominant at proestrus stage when estrogen hormone increases. At estrus stage, cornified squamous epithelial

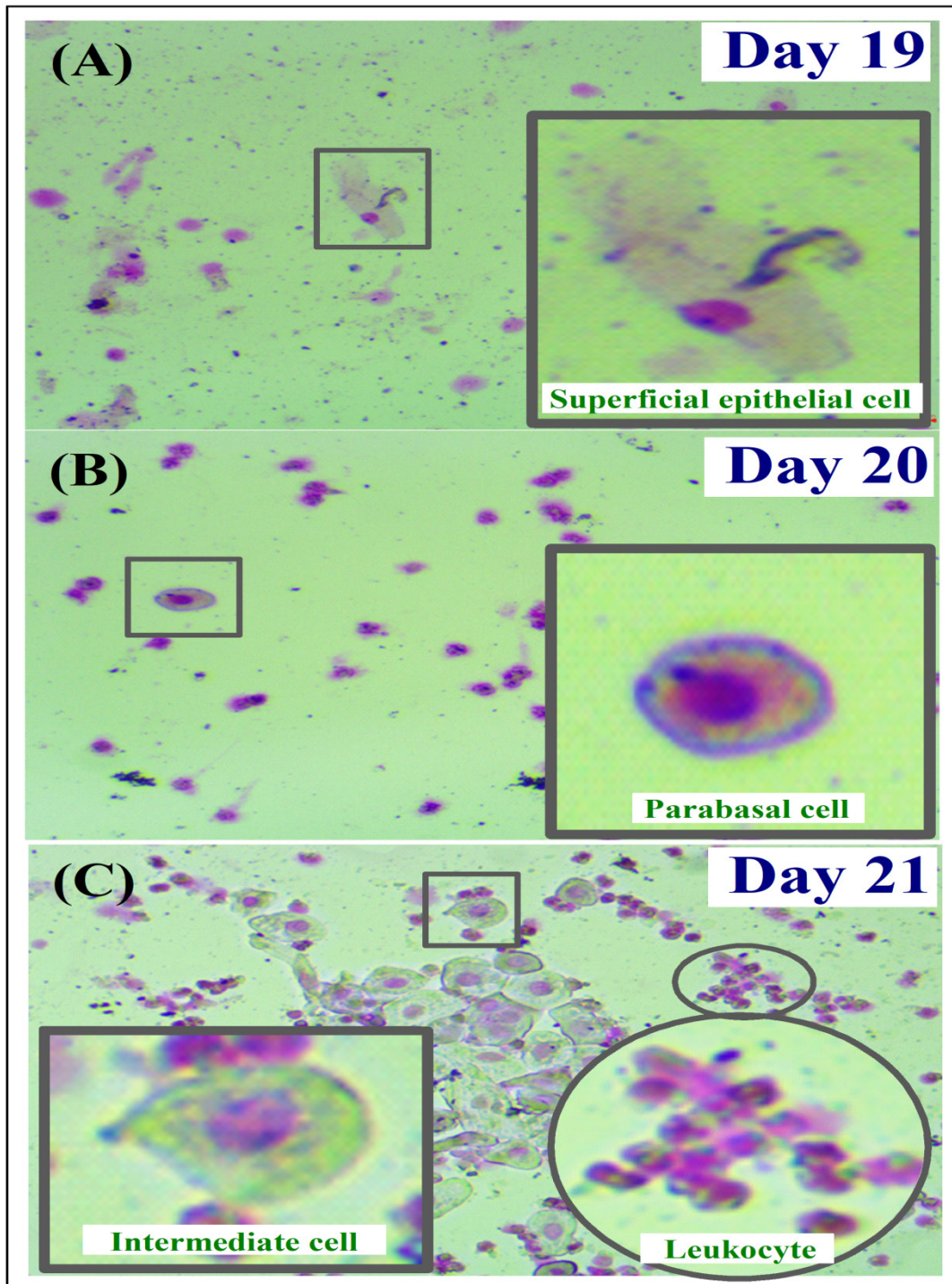


Figure 1. The characteristics of vaginal cells in pregnant buffaloes after 19 to 21 days of artificial insemination.

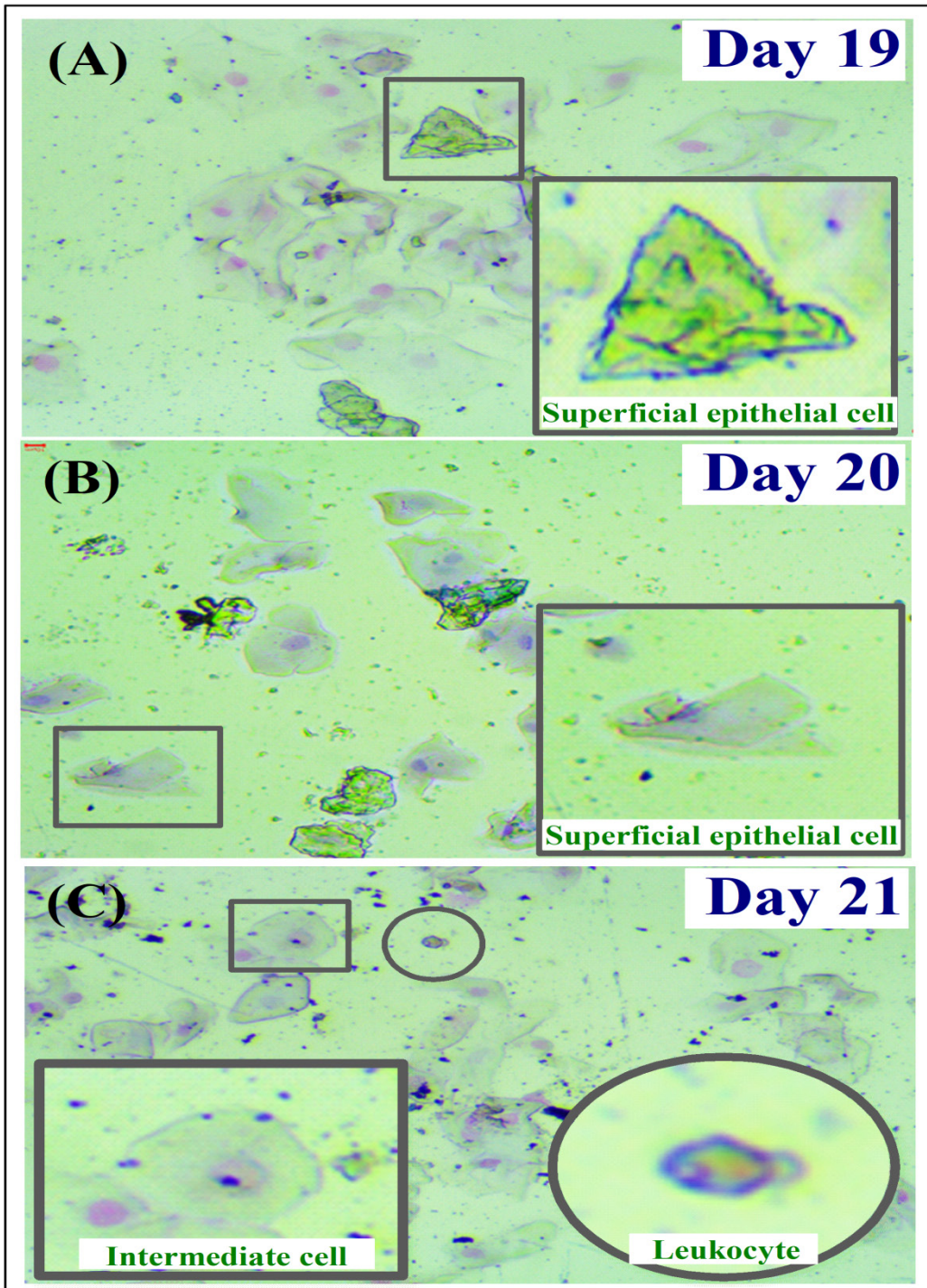


Figure 2. The characteristics of vaginal cells in non-pregnant buffaloes after 19 to 21 days of artificial insemination.

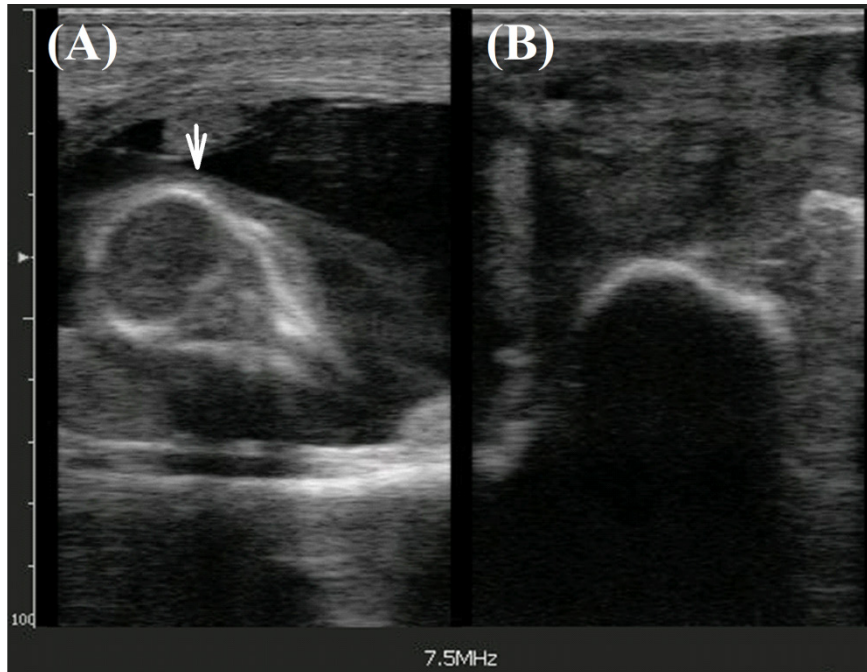


Figure 3. Using ultrasonography to confirm the pregnancy on day 90 after artificial insemination, (A) pregnant buffalo and (B) non-pregnant buffalo.

Table 1. Comparison between two protocols of estrus synchronization on the percentage of pregnancy.

Estrus synchronization protocols	No. of animals	Pregnancy rate (%)
GnRH + PGF2 α + GnRH	17	58.82 (10/17) ^a
GnRH + PGF2 α	13	84.61 (11/13) ^a

Different superscripts indicate significant differences among treatments ($P < 0.05$).

Table 2. The comparison of pregnancy diagnosis method between vaginal smear and rectal palpation and ultrasonography.

Method of pregnancy diagnosis	No. of Animal	Pregnancy	Non-Pregnancy
Vaginal Smear	30	18	12
Rectal palpation and ultrasonography	30	21	9
%Accuracy vaginal smear	30	85.71	66.76
%Accuracy rectal palpation and ultrasonography	30	100	100

cells without visible nuclei present in clusters. At metestrus stage, a cell mixture with leucocytes and a few nucleated epithelial and/or cornified squamous epithelial cells present when plasma estrogen concentration is low. At diestrus stage, leukocytes are dominant since estrogen hormone levels start to increase (Caligioni, 2009a). In the present study, the proportion of exfoliative vaginal cells in cattle during various stage of estrus cycle was observed using vaginal cytology. The results showed that the proportion of superficial cell was higher at the estrus period because estrogen hormone increases the activeness of uterus wall. It causes hypersecretion in epithelial cells of the uterus and vagina, and therefore the superficial cells followed the vaginal peel (Siregar *et al.*, 2016). In goat, the superficial cells were more frequently observed, and they appeared to be associated with the proestrus, estrus, and early metestrus phase of the cycle (Ola *et al.*, 2006). The intermediate and parabasal cells presented at diestrus phase which correspond to luteal phase controlled by progesterone (Ola *et al.*, 2006). Therefore, we suspected that the different of the morphology of exfoliated cells of vaginal smears between pregnant and non-pregnant swamp buffaloes may be associated with estrogen and progesterone hormones. Moreover, the status of pregnancy and non-pregnancy were confirmed and noted using rectal palpation and ultrasonography on day 90 after AI. The correct pregnancy or non-pregnancy diagnosis of swamp buffaloes were confirmed by both ultrasonography and rectal palpation. In the scanning, positive diagnosis of pregnancy was based on the presence of the amniotic and allantoic vesicles as non-echogenic cavities closely surrounding the embryo on day 90 after AI (Figure 3A). A total of thirty buffaloes were scanned for pregnancy diagnosis on day 90 after AI. The

results showed that twenty one buffaloes were pregnant and nine buffaloes were not pregnant. The accuracy of rectal palpation and ultrasonography for pregnancy diagnosis was 100% (Table 2).

CONCLUSION

Vaginal smear technique is one alternative method to determine early pregnancy because it is a simply method and low cost. Importantly, this technique can help us to determine early pregnancy on day 19 to 21 after AI and can reduce the problems of early pregnancy diagnosis in swamp buffaloes.

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