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Non-invasive clinical diagnosis of estrus for AI synchronization using vaginal cytology in three bubaline breeds in the Philippines

Peregrino Duran[1], Hilarion Karlo V Corpuz[2], Dianne Camille A Gaspar[2], Charisse M Misola[2], Madison P Munar[2]* and Danilda Hufana-Duran[1]

[1]Philippine Carabao Center, National Headquarters and Gene Pool, Science City of Muñoz, Nueva Ecija 3121, Philippines
[2]Department of Biology, College of Science, University of the Philippines Baguio, Governor Pack Road, Baguio City 2600, Philippines

ABSTRACT

Artificial insemination is a reproductive technology commonly employed in the genetic improvement program on water buffaloes in the Philippines. However, conception rate with artificial insemination is still hampered due to low reproductive efficiency of these animals. In this study, estrus cornification index was investigated in three bubaline breeds namely, Native Philippine Carabao (NPC), Bulgarian Murrah (BuM) and Brazilian Murrah (BrM) to develop a method that will increase heat detection and synchronize administration of artificial insemination in field conditions. A conception rate of 100% after artificial insemination was observed on each of the breeds inseminated with cornification index within the range 46% to 66%, 39% to 64%, and 55% to 67% in NPC, BuM, and BrM, respectively. A cornification index within the specified range reinforced with the natural estrus signs was observed to increase artificial insemination success rate in the three bubaline breeds.

Keywords: artificial insemination, cornification index, Native Philippine Carabao, Bulgarian Murrah, Brazilian Murrah

Corresponding author

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INTRODUCTION

Water buffaloes have been an integral part of the Philippine agricultural industry. They are commonly used for draft power, milk and meat production (Nanda and Nakao, 2003; Mondal, 2007). Cross breeding of imported riverine buffaloes with native Philippine buffaloes was being done in order to improve the genetic characteristics of native buffaloes (Philippine Carabao Center, 2008). However, one of the main limitations in improving the genetic pool of water buffaloes is the live animal importation for cross breeding which is laborious and expensive (Hufana-Duran,2008). Artificial Insemination (AI) is usually done to address the problem on live animal importation (Hufana-Duran, 2008). There is a low AI success rate observed in water buffaloes (IAEA, 2005, Pomares, *et al.*, 2012). The long intercalving period is the main problem in improving the genetic characteristics of these animals (Nam, 2010). Another is that these animals are shy breeders and cannot fully express their heat (estrus) (Barile, 2005). Acceptance of the male buffalo is considered to be the most reliable indication of estrus (Barile, 2005). Weak expression of reported clinical indicators such as frequent urination, bellowing, vulvar swelling, and mucus discharge, also adds to the low heat detection on these animals (Barile, 2005).

The success rate of AI is therefore being limited by the weak detection of estrus with the aid of the current clinical indicators. The success rate of AI may be improved through employing the techniques in vaginal cytology. Vaginal cytology is a type of endocrine assay that tracks the changes in the morphology of the epithelial cells during estrous cycle. Vaginal cytology was established as a good clinical indicator in small ruminants, however, this was not yet done in water buffaloes. Investigation on vaginal smears by observing the changes in morphology of the vaginal epithelial cells through the progression of estrus, can eventually identify the perfect timing for AI. This study aimed to establish a non-invasive clinical indicator based on the estrus cornification index to reinforce heat detection and improve reproductive efficiency in water buffaloes.

MATERIALS AND METHODS

This study was carried out on three different breeds of water buffaloes namely; Native Philippine Carabao (NPC, 4 heads), Brazilian Murrah (BrM, 6 heads) and Bulgarian Murrah (BuM, 3 heads).The animals were randomly selected irrespective of parity, milk yield, body condition and weight. The animals were injected with prostaglandin (PGF2α, two ml of Bioestrovet® or five mL of Lutalyse®) to induce estrus. Vaginal cytology was carried out by observing the changes in the vaginal smears from the day of prostaglandin injection until the end of AI service. Vaginal examination was carried out on a daily basis in a span of five to six days until these animals exhibited visible estrus symptoms and subjected to AI. Animals recorded in heat after three days were artificially inseminated. All animals were then diagnosed for pregnancy via rectal palpation approximately 60 days post AI and confirmed after 120 days.

Preparation and Staining of Vaginal Smear

Prior to swabbing, the vulva was cleaned using tap water and a tissue paper. A six-inched sterile cotton swab was inserted into the vaginal opening, ensuring that the swab was inserted several inches past the vulva. Once the swab was fully inserted, it was rotated at least 90° to 180° gently against the vaginal wall, leaving few seconds to allow the animal to relax and was then withdrawn gently. This was done to ensure that the cotton swab was able to acquire adequate load of cells needed for observation. The smears were prepared immediately after the withdrawal of the swab, gently rolling the swab along the length of the glass slide. The smears were fixed by dipping it to Carnoyl Fixative. After fixing, the slides were allowed to dry before staining with KARYOMAX® Giemsa Stain (Life Laboratories, USA). Optimized time of staining was determined to avoid under and over staining of the smear. Three minutes staining with Giemsa stain produced the most ideal specimen for microscopic observation. The stain was regularly replaced and filtered with 0.22µ membrane to avoid contamination with vaginal cells from previous preparations. The vaginal epithelial cells were classified upon examination of smears under compound light microscope (LEICA) using oil immersion microscopy. Each slide was examined for the following features: the presence and number of cornified epithelial cells and nucleated epithelial cells (parabasal, early intermediate, late intermediate). The process of determining the phase of estrous cycle was done according to the type of cells that

are present in the smear. At least 200 cells per slide were examined and classified. Data from each sample were treated independently and the observed changes in epithelial cells were then correlated with the observed external animal behavior and manifestations of estrus during the vaginal sampling and with the result of the AI.

Artificial Insemination

Artificial insemination was carried out after inducing estrus. Frozen semen from two proven bulls was used. Frozen semen was thawed at 37°C for 15 seconds, loaded in AI gun and deposited in the uterus. The vaginal epithelial cells and cornification index sampled before and after the AI period was correlated to the conception rate recorded 60 days after AI service.

RESULTS AND DISCUSSION

Comparison of Vaginal Cytology

The cells were classified primarily on the size of the nucleus relative to the size of the cytoplasm. In relation with the past studies conducted in canine (Rottweilers, 2000; Reddy *et al.,*2010), goats (Safiriyu *et al.*, 2005), and rats (Singletary *et al.*, 2005), three classifications of vaginal epithelial cells which include parabasal, intermediate and cornified cells were established. In contrast, in this study we further categorized intermediate cells into early and late stages to provide a clearer illustration on the transition of the epithelial cells with respect to the stage of the reproductive cycle. Early intermediate cells were characterized by having low nucleus to cytoplasm size ratio. Late intermediate cells were characterized by disintegrating nucleus indicating that the cells are undergoing cornification.

The parabasal cells of NPC were observed to be more differentiated, having a distinct rounded nucleus, as compared to the parabasal cells of BuM and BrM with nucleus having grainy appearance. Noticeably, the parabasal cells of BrM are relatively larger in size than the other breeds. Consequently, early intermediate cells of NPC were observed to be more differentiated, wherein the nucleus and the cytoplasm can easily be distinguished as compared with BuM and BrM (Ayen *et al*., 2012). There is hardly any distinguishable difference in the late intermediate cells among the different breeds apart from the relatively bigger cell size in BuM. The margin of cornified epithelial cells in NPC appears to be more regular in contrast with the cells of BuM and BrM which appears to be rougher on its margin.

It was observed that when animals are not in estrus there is predominance of parabasal cells over intermediate and cornified cells (Table 1, Table 2, Table 3). Along with parabasal cells, early intermediate cells decreases as the animal shifts to estrous period. The progression of the stages to estrous shifted the predominance to cornified cells, this result conformed to the report by Post (1985). Sudden increase in the number of the cornified cells was observed as the animals responded to the prostaglandin. The cyclic cellular changes in the vaginal epithelium occur as a result of the change in secretory patterns of reproductive hormones as observed by Mshelia *et al*. (2001). It was indicated that changes in hormone concentration in blood such as sex steroids, specifically estrogen in the ovary, determine the changes underwent by the vaginal epithelium throughout the cycle. Cornification of vaginal epithelium results due to the increase in levels of estrogen. During cornification, the surface of the epithelium becomes large and flattened and nucleus is disintegrated which supports earlier observations (Bowen, 1998).

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Table 1: Distribution of vaginal epithelial cells before and after Artificial Insemination in Native Philippine Carabao (NPC).

Day	Parabasal (%)	Early Intermediate (%)	Late Intermediate (%)	Cornified cells (%)	Total cell count	Remarks
1	27.73	25.48	15.94	30.85	577	Estrus synchronized
	29.73	24.81	17.80	27.65	528	
2	11.57	8.26	16.53	63.64	608	No signs of estrus
	14.68	11.24	16.74	57.34	436	
3	9.47	19.66	15.78	55.10	824	No signs of estrus
	12.87	16.58	17.81	52.73	567	
4	8.28	20.76	22.29	*48.66	785	Mucus discharge, AI administered
	12.29	16.54	24.28	*46.89	659	
5	5.53	15.45	13.66	*66.37	615	Mucus discharge, tail raising, standing estrus, AI administered
	8.83	17.35	10.41	*63.41	317	
6	53.63	15.93	9.13	21.31	427	No signs of estrus
	42.65	10.98	2.28	44.08	701	

*Animal diagnosed pregnant 60 days after AI.

Table 2: Distribution of vaginal epithelial cells before and after Artificial Insemination in Bulgarian Murrah (BuM).

Day	Parabasal (%)	Early Intermediate (%)	Late Intermediate (%)	Cornified cells (%)	Total cell count	Remarks
1	9.89	14.11	22.95	53.05	475	Estrus synchronized
	8.83	13.56	22.40	55.21	475	
2	8.44	14.11	19.17	58.28	652	No signs of estrus
	8.89	13.89	19.81	57.41	540	
3	6.05	7.99	22.25	63.71	463	Standing estrus
	6.26	14.09	19.13	60.52	575	
4	6.52	9.35	22.83	*61.30	460	Frequent urination, tail raising, mucus discharge, AI administered
	7.21	8.35	19.73	*64.71	527	
5	21.94	18.46	18.46	*41.15	661	Frequent urination, tail raising, AI administered
	27.47	19.37	14.03	*39.13	506	
6	36.64	21.92	9.93	31.51	292	No signs of estrus
	38.63	24.88	9.00	27.49	422	

*Animal diagnosed pregnant 60 days after AI.

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Table 3: Distribution of vaginal epithelial cells before and after Artificial Insemination in Brazilian Murrah (BrM).

Day	Parabasal (%)	Early Intermediate (%)	Late Intermediate (%)	Cornified cells (%)	Total cell count	Remarks
1	16.43	21.55	48.76	13.25	566	Estrus synchronized
	18.86	20.36	45.51	15.27	334	
2	4.71	8.38	38.74	48.17	191	Mucus discharge
	8.74	6.99	32.52	51.75	286	
3	4.81	13.52	13.70	*67.96	540	Mucus discharge
	4.16	16.64	14.56	*64.64	625	AI administered
4	21.37	16.91	5.74	*55.98	627	Mucus discharge,
	20.69	16.01	5.17	*58.13	406	AI administered
5	71.94	7.91	2.52	17.63	556	No signs of estrus
	74.05	8.16	2.04	15.74	343	
6	68.50	23.10	2.62	5.77	381	No signs of estrus
	70.83	20.83	3.85	4.49	312	

*Animal diagnosed pregnant 60 days after AI.

Increased AI Efficiency

The range of cornification indices during the time of AI was recorded in three buffalo breeds. Among the four NPC, three animals showed estrus signs after estrus synchronization. These animals were artificially inseminated within the cornification index that ranges from 46% to 66%. One out of three animals in BuM showed estrus signs after estrus synchronization and these animals were artificially inseminated within the cornification index that ranges from 39% to 64%; and three out of four BrM within the range of 55% to 67%. The administration of AI within the identified range of cornification indices yielded a 100% conception rate on all the three water buffalo breeds. The high cornification index among the three buffalo breeds was observed to be a good clinical indicator of estrus to identify the perfect timing of AI.

CONCLUSION

Physical manifestations of estrus were observed to occur within and outside the actual estrus stage. Approximation of perfect timing to administer AI can be achieved through concurrent examination of the relative percentages of the cells present in the vaginal smear with the physical manifestations of estrus. Based on the data gathered, high percentage of parabasal cells is a characteristic of diestrus stage when the animal resists vaginal penetration and displays no physical manifestations of estrus. As the cycle continues, it was observed that the cells begin to proliferate, differentiate and exfoliate, resulting to increased number of cell layers, rapidly changing the parabasal cells into intermediate, and then to cornified cells. This study have shown that cornification index can also be used as potential non-invasive clinical indicator of estrus in NPC, BuM and BrM. High cornification index does not necessarily indicate that the animal is in estrus. It should be noted that high cornification index reinforced with salient estrus signs can facilitate the identification of perfect AI timing thus significantly increasing its success rate.

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