Ovozoa 14 No. 1, April 2025 p-ISSN 2302-6464 e-ISSN 2722-967X
Available at https://e-journal.unair.ac.id/OVZ/index

Original article

Performance of pre-breeding cycle-staging techniques in bitches: Clinical comparability of vaginal cytology and progesterone assay

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Open access under CC BY – SA license, DOI: 10.20473/ovz.v14i1.2025.1-7
Received March 5 2024, Revised November 30 2024, Accepted December 3 2024
Published online April 2025

ABSTRACT

Accurate timing of insemination (mating) remains a critical challenge in the reproductive management of bitches. In spite of technical advances in vaginal cytology (VC) and serum-progesterone (SP) assay, the challenges appeared not to have abated. Five clinical scenarios involving VC and SP are presented, aimed at investigating technique-performance and creating more robust awareness on application of SP titers obtained using a fluorescence immunoassay kit having 7.6–20.0 ng/mL as predictable-progesterone-titer-range-for-insemination-(PPTRi). Number of bitches, insemination-readiness-detection-technique(s) (IRDT), score (%) of agreement/disagreement between Insemination-readiness-detection techniques (SABT) for peculiarities of bred bitch(es) (PBB), proportion of bitches that became pregnant confirmed by relaxin assay and, number of pups littered were studied. Results obtained for number of bitches, IRDT, SABT or PBB, relaxin and number of pups littered, respectively were: 22, VC-SP, 81.8%/18.2%, 72.7% and, NA (i.e. not-available) (case I); 2, VC-SP, bitches mated after attaining PPTRi 10 and 13 days after been detected ready-for-mating via VC, 100%, and, 5 and 7, respectively (case II); 1, SP, bitch mated at SP of 4.401 ng/mL, 100% and, 11 (case III); 2, SP, first bitch mated at 4.682 ng/mL and the second at 7.049 ng/mL, 100% and, 9 and 12, respectively (case IV); 1, VC-SP, bitch mated once detected ready-for-mating by VC while SP at 24-hours post-mating was 2.852 ng/ mL, 100% and, 1 (case V). These findings suggest high-level agreement in accuracy of Insemination-readiness-detection between the two techniques, with none reflecting superiority. For better breeding successes, it is recommendable to inseminate earlier, during the peri-ovulation period.

Keywords: Agreement, bitch, breeding, detection techniques, peri-ovulation

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INTRODUCTION

The dog business industry is no doubt highly promising in terms of revenue generation, as it allows for imports and exports, thereby possessing huge capacity for job creation and boosting of national economy. This revenue-generation depends a lot on the extent to which the fertility potential of canine sex partners is maximized. The peculiarities of the estrous cycle in bitches allow for frequent and, probably avoidable errors in breeding management. In order to reduce/obliterate these errors/limitations, numerous studies have reported easy-to-use estrus detection methods and aids such as vaginal cytology (VC), vaginal electrical conductance probes, progesterone vaginal ovulation test strips, saliva/vaginal fern pattern mini-scopes, quantitative progesterone assay (PA) kits, among others. The use of these techniques had been associated with varying degrees of successes and failures hence, a recent report (Pavlo *et al*., 2022) advocated that multiple techniques should be used for a more effective determination of breeding time in single bitches in order to enhance fertility and productivity. In Nigeria and most parts of sub-Saharan Africa, vaginal exfoliated studies (VC) appear to be the commonest tool employed by veterinarians and other stakeholders in determining the best time to breed bitches, either naturally or artificially (Obafemi *et al*., 2021).

Vaginal cytology is based on the characteristic nature of the vaginal epithelium, which is dynamic in its cellular pattern and in response to cyclical changes in endocrine pattern (Leigh *et al*., 2013). Vaginal cytology has thus been proven as a reliable technique in staging the estrous cycle in bitches as well as some other mammalian species. From some of our recent studies however, we reported the increasing limitation of VC in effectively staging the bitch cycle and most importantly, determining the critical breeding time (Obafemi *et al*., 2021). Part of the observed limitations included a wide window during which the bitch continues to show cytological readiness for mating, parading superficial epithelial cells between 75 and 99% of total exfoliates. There were also extreme instances where the cytological picture of vaginal exfoliates remained poorly diagnostic, not showing any characteristic epithelial cellular architecture that are depictive of any cycle stage.

During instances as these, a parallel technique of quantitative PA has shown its superiority in clinical prediction of optimum breeding time in such bitches, with good pregnancy outcomes (Akinbote *et al*., 2022). The kit used has three predictable progesterone titer-ranges for insemination. The significance of this paper lies not only in that it reports some awful observations with certain bitches whose serum were subjected to quanitative PA for the determination of their mating time but also that, the frequency of such occurrences appears to be on the increase, hence, breeders and veterinarians need be on the watch.

Case report

Five clinical scenarios are reported in this paper as follows:

Case I:

Between August and September, 2022, blood samples of a total of twenty-two bitches (multiple breeds) were received, from 2 major stud stations, for progesterone confirmation, on stud owners' insistence, of readiness for mating following advice, ascertained via serial VC from notable Veterinary establishments located in Ibadan, Nigeria.

Case II:

Blood samples from two (a Caucassian and a Boerboel) adult bitches (more than one parity) were presented on 24/06/2023 for PA. Both bitches were earlier presented for VC at separate clinics and the respective clients were instructed to proceed with artificial insemination on 24/06/2023. The progesterone titers were 0.212 ng/mL (Caucassian) and 1.183 ng/mL (Boerboel). Following the assay, the clients were advised to present the bitches for retest between 3 and 5 July, 2023.

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Case III:

An adult Caucassian bitch was accidentally mated by another stud on the day her serum yielded a progesterone titer of 4.401 ng/mL. The client suddenly became occupied that he could not keep subsequent appointments for retest as the bitch had not attained the predictable progesterone titer-ranges for insemination (PPTRi). The client returned to the clinic 6 weeks after the progesterone test only for reproductive examination to confirm she was pregnant. The bitch later whelped eleven pups.

Case IV:

Two adult German shepherd bitches showed serum progesterone titers of 4.682 ng/mL and 7.049 ng/mL respectively on the day they were presented in the clinic. Both were mated only once on that same day.

Case V:

An adult Caucassian which had been mated at consecutive estrus since she littered 3 years earlier was presented for PA, which yielded 2.852 ng/mL on 28/04/2023. Prior to this, on 26/04/2023, the bitch was inseminated as recommended by a clinician who had carried out VC on her. Repeat progesterone assay yielded 6.827 ng/mL and 6.024 ng/mL on 03/05/2023 and 06/05/2023, respectively. Repeat VC (at progesterone point) on 06/05/2023 showed very good but sparse superficial epithelial cells with folding. Blood and vaginal swab were collected on 06/06/2023 for haematology and, culture and sensitivity, respectively. Major report from the laboratories included: Chronic inflammatory/infectious condition with a heavy, multiple bacterial infection. Based on these findings, the bitch was treated with Doxycycline (10 mg/kg) followed by immune boosters. While the next heat was being awaited, the bitch unexpectedly whelped a stillbirth on 07/07/2023.

MATERIALS AND METHODS

Data collection and summarization

Data were extracted from the database for all bitches whose sera were submitted for SP testing for the determination of their mating/insemination time. Standard practice of the laboratory included telephone/physical follow-up on all cases presented to the clinic. Data were summarized using descriptive statistics.

Progesterone assay procedure

Intravenous blood (2 mLs) was collected at different times from each bitch beginning from the point of presentation of the bitch for examination.

The blood was allowed to clot for some minutes after which it was centrifuged at 4000 rpm for 15 minutes for the serum to be collected. Following venous blood collection and serum separation, progesterone titer was determined via Fluorescence Immunoassay using the MSL-YT02 kit (Medsinglong Co. Ltd. China). The kit has 3 PPTRi as follows: 7.6-8.5 ng/mL (inseminate after 24 hours), 8.6-9.9 ng/mL (inseminate after 12 hours) and 10.0-20.0 ng/mL (inseminate immediately).

Vaginal cytology procedure

Following restraint by an assistant, a standard sterile vaginal swab stick, aided by a speculum, was gently inserted, into the anterior vagina to collect the smear. Further processing was done following standard protocol (Aydin *et al*., 2011; Gurumyen *et al*., 2021). Exfoliated cells were thereafter viewed under the microscope at x400.

Pregnancy diagnosis

Serum sample from each bitch was tested for relaxin using the WITNESS rapid test kit (Zoetis Inc. Kalamazoo, France). Using the provided dropper, 2 drops of serum were carefully placed in the reagent hole and incubated for 15 minutes. Result was read as follows: appearance of red lines on T and C (positive), appearance of red line on T only (invalid) and, appearance of red line on C only (negative). Litter size was also recorded at the point of whelping in each bitch.

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RESULTS

Case I:

The progesterone titers of eighteen (81.8%) out of the twenty-two bitches had values that fell within the PPTRi of the kit used. Furthermore, only sixteen (72.7%) bitches were thereafter confirmed pregnant and all whelped safely.

Case II:

Both bitches attained PPTRi between 10-13 days after which they should have been inseminated, going by VC. Both bitches were confirmed pregnant via relaxin assay conducted post-insemination.

Case III:

The client returned to the clinic 6 weeks after the progesterone test only for reproductive examination to confirm she was pregnant. The bitch later whelped eleven pups.

Case IV:

Both bitches were confirmed pregnant by relaxin assay conducted four weeks post mating. First bitch mated at 4.682 ng/mL and the second at 7.049 ng/mL, 100%. The bitches whelped 9 and 12, respectively.

Case V:

The bitch unexpectedly whelped a stillbirth on 07/07/2023, while the next heat was being awaited.

Table 1 Particulars of case I

number of bitches involved	mating readiness detection techniques used	score (%) of agreement/ disagreement between techniques	proportion of bitches that became pregnant/'n' pups
22	VC and PA	81.8%/18.2%	72.7%/NA

VC: vaginal cytology; PA: progesterone assay; NA: not available

Table 2 Particulars of case II, III, IV, V

number of bitches involved	mating readiness detection technique used/value	peculiarity of bitches with respect to modalities of detection technique	proportion of bitches that became pregnant/'n' pups
2	VC and PA	1st and 2nd bitches attained PPTRi on day 10 and 13, respectively after they had been detected ready for mating via VC	100%/NA
1	PA/4.401 ng/mL	Bitch was accidentally mated on day of PA, whereas PPTRi recommendation is 7.6-20.0 ng/mL	100%/11
2	PA/4.682 and 7.049 ng/mL	Bitch was accidentally mated on day of PA, whereas PPTRi recommendation is 7.6-20.0 ng/mL	100%/21
1	VC and PA	Bitch was mated once as recommended by VC. On days 2, 6 and 9 after, SP (ng/mL) were 2.852, 6.827 and 6.024, respectively. Repeat VC on d9 showed very good but sparse superficial epithelial cells with folding. Sequel to Laboratory findings, bitch was administered Doxycycline (10 mg/kg) and immune boosters	100%/1 (stillbirth, day 73 post-mating)

PA: progesterone assay; PPTRi: predictable progesterone titer-range for insemination; SP: serum progesterone; VC: vaginal cytology; NA: not available

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DISCUSSION

Progesterone testing for the determination of best mating time for bitches is not only popular among dog breeders but has scientific backing from the standpoints of research and clinical practice. Although, the technique appears to be poorly available in many African communities, probably due to cost or awareness, one recent report from Nigeria ascertained that subjecting bitch to PA prior to breeding resulted in higher pregnancy outcomes which is capable of eventually upscaling dog breeding business (Labib *et al*., 2018; Akinbote *et al*., 2022).

Part of the findings in cases I and V appears to lend credence to the reliability of VC as an effective technique for staging the cycle in bitches. The involvement of PA in case I underscores a basis for comparison of dependability and agreement or disagreement between VC and PA in the study. That a high proportion of the bitches detected, via VC, as ready for mating were confirmed by PA indicate a substantial good level of agreement between the two techniques. However, the less than 20% disagreement also suggest some query on the dependability of the two techniques. This query is apparently difficult to unravel within the limits of the present study for many reasons. The fact that the proportion of pregnancy which resulted was lower to the agreement level between the techniques indicates the importance of other key determinants of pregnancy apart from accuracy of estrus detection and timing of insemination. Again, the present results may not suffice to attach superiority to either technique but if it may, the lone instance of case V, if put in perspective with the level of agreement in case I, may support VC as the more dependable of the two for determination of mating time in bitches.

Case III presents an interesting situation which raises much clinical concern, with regards to the reliability/dependability of SP assay, for breeders and veterinarians. Following deposition of the ejaculate in the female tract, the best spermatozoa are not known to survive beyond 7 days in most mammalian species, their survival being subject to various factors including nutrition and health of the male, sperm viability as well as utero-vaginal factors (Rijsselaere *et al*., 2014; Saint-Dizier *et al*., 2020). There has been reports of spermatozoa surviving in the reproductive tract of the bitch for about 9 days. In order for these spermatozoa to remain functionally competent, they are stored temporarily in reservoirs such as the uterine crypts and glands, and at the glandular mounds of the distal part of the uterotubal junction (England *et al*., 2013; Rijsselaere *et al*., 2014).

The canine estrous cycle relatively has a longer duration of most stages compared with food animals. The bitch is also peculiar in that many do allow mating in their late proestrus (Silva *et al*., 2020; Nesa *et al*., 2022) compared with other domestic female animals which allow mating only during estrus. These peculiarities allow the bitch to have a longer window, often exceeding seven days, during which mating activities may occur. The clinical relevance of the PPTRi must be appreciated as descriptive of the status of the oocyte, in terms of viability or age *vis a vis* spermatozoa binding that would result in conception. In view of the comparatively large litter size obtained in this situation (11pups), it is not unlikely that the mating occurred, early enough before the oocytes ripened, by a stud whose spermiogram must have been excellent.

While this scenario may always be the desire of practitioners, the clinical concern is that the mating occurred outside all of the recommended PPTRi. The litter size recorded in this case is also comparable to that of a recent study conducted in the same locality in which a range of 5-10 puppies/bitch was reported (Akinbote *et al*., 2022). Other researchers have reported similar results from intrauterine artificial inseminations carried out 8 or 9 days post LH surge in bitches. This resulted in 60% pregnancy rates (England and Concannon, 2002). They also reported that the peak of fertility in natural mating in the bitch is between 2 days prior to ovulation until 4 days after ovulation.

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There was no difference in pregnancy rates and liter sizes following singular mating in this window. Noting that in the work of Akinbote *et al*., (2022), breeding followed PPTRi recommendations, the outcome of case III may then suggest that insemination outside these recommendations are not in any way less superior or effective with regards to conception and litter size. While it is advisable that practitioners keep to precautions of scientific procedures, it remains unclear how a single mating which occurred far below the least PPTRi not only resulted in conception but produced a good litter size. Usually, progesterone titers above 3.0 ng/mL have been known to indicate ovulation in bitches (Kunanusont *et al*., 2021). Schmicke *et al* (2016) also have detected SP titers of 2-4.5 ng/mL on the day of ovulation in bitches.

Post-ovulation, the eggs are also known to ripen for fertilization in about 2.5 to 3 days (Astudillo *et al*., 2023). It is not unlikely that observation with case III is enigmatic, in which the progesterone titer rapidly increased after that day's assay or the ovulated oocytes underwent precocious maturation. Whichever way it is looked at, it may be expedient to advise practitioners not to indulge under the circumstances of case III as the consequences may be anti-productive. Moreover, the history in case II appears to provide a basis to compare superiority of the two techniques involved in this study. Had the sample size been larger, it would have clearly underscored the challenges with the use of VC which may have also insipidly/faintly played out in cases I and V. In a recent study by Obafemi *et al*., (2021), authors reported that parameters of VC i.e. proportion of superficial epithelial cells and clumping extent appeared to be effective in determining ovulation in only about 70% of bitches sampled. This report, combined with observations in case II strongly suggests that serum PA is superior to VC in timing insemination in bitches.

CONCLUSIONS

For better breeding successes, it is recommendable to inseminate earlier, during the peri-ovulation period, as the predictable-progesterone-titer-range-for-insemination appears to safeguard delayed breeding interventions majorly.

ACKNOWLEDGEMENT

Authors appreciate the support and cooperation of Ibadan Dog Breeders Association.

AUTHOR'S CONTRIBUTIONS

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SAF, OMO: conceived the idea, designed the overall structure of the manuscript, and drafted the manuscript. TAA, BAS, MBR: acquired, analyzed, and interpreted the data. ORA, AOO, DBO, OOF, OOL: critically reviewed and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest to declare.

FUNDING INFORMATION

This study was funded by the authors.

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