Research brief max 2000 words

ADAPTATION OF A POINT-OF-CARE CANINE PROGESTERONE TEST FOR USE OF PARTURITION PREDICTION IN CAPTIVE ASIAN ELEPHANTS (*Elephas maximus*) - PROOF OF CONCEPT

Fieke M. Molenaar, DVM, MSc, DZooMed, MRCVS, Marcus Rowcliffe PhD, and Andrew Lakey PhD

From ZSL Whipsnade Zoo, Zoological Society of London, Dunstable, LU62LF, UK, fieke.molenaar@zsl.org (Molenaar); Institute of Zoology, Zoological Society of London, Regents Park, NW14RY (Rowcliffe): RQM+, Blenheim Court, Huntingdon Street, Nottingham, NG1 3BY, United Kingdom UK (Lakey).

Abstract: In the Asian elephant (*Elephas maximus*), progesterone products 5α-pregnane, 3α-hydroxypregnane and 17α-progesterone can be measured as a predictor of impending parturition[2]. Measurement of a sudden decrease in blood progesterone levels is considered one of the most objective ways to predict impending parturition in elephants, and previous trends of serum progesterone in the final stages of ten elephant pregnancies at the study collection showed that the pregnancy progesterone values halved approximately five days prior to actual parturition. Point of Care (POC) tests eliminate the cost involved in transporting samples to an external laboratory and provide an almost instant result, facilitating decision making with regards to animal monitoring and management. The aim of this study was to investigate the ability of the AgPlus Point of Care Immunoassay System to measure 4-pregnen-3,20-dione in pregnant elephant samples and adapt the

method for detection of the pre-parturient progesterone decrease. Frozen serum samples of two pregnant elephants (N = 86), and fresh serum samples of one pregnant elephant (N = 11) were analysed using both the POC method and a radio-immuno-assay in a reference laboratory. Statistical analysis of the data showed that there was no significant difference between the two methods for detection of the progesterone drop, indicating that the POC method can be considered appropriate for use in elephant parturition prediction.

INTRODUCTION

Elephants have the longest gestation of any mammal, with published times ranging from 637 to 686 days in Asian elephants (*Elephas maximus*) [11]. At the study collection, the mean gestation length with a known mating date is even longer at 661 days (N = 10, range 630-700 days). Parturition prediction based on gestation length is therefore not accurate. Although parturition prediction is possible through recording of behavioural and visible changes, the most accurate and objective way is by i) the measurement of blood progesterone products (such as 5α-pregnanes[7] and 17α-hydroxy-progesterone[11,15]), showing a drop to baseline at parturition[2,9], and ii) transrectal ultrasonographic visualisation of dilation of the cervix, the latter giving a 12-24h warning of impending parturition[6]. To be able to detect this sudden drop in serum progesterone products, analysis of serial blood samples during the later stages of pregnancy will determine pregnancy levels. As a drop in progesterone products can be followed by parturition within one day[2-4], swift same-day analysis of the samples is ideal.

Point of Care (POC) tests are diagnostic tests that can be carried out near the patient. A variety of biochemical analysers have been developed and improved for use in standard veterinary practice[1,5,16]. Several POC tests are available for reproductive hormone measurements in veterinary medicine, including for instance the recent development of

measurement of progesterone in the milk of cattle[17] , but as yet no POC test has been developed for the measurement of elephant progesterone products.

The aim of this study was to adapt and validate a recently developed rapid, quantitative diagnostic platform technology for use in POC applications, the canine POC Progesterone Immunoassay System (AgPlus Diagnostics Ltd, The Exchange, Colworth Science Park, Sharnbrook, Bedford, UK) for the use of blood progesterone measurements in Asian elephants, to facilitate fast, reliable and cost-effective in-house blood progesterone measurements in the final stages of pregnancy, assisting accurate parturition prediction. For this approach to be valid, we would expect that blood progesterone product concentrations measured using the AgPlus canine progesterone POC assay to correlate closely with concentrations measured using a reliable laboratory-based radio-immune-assay, or less stringently, that the two assays should at least detect pre-partum falls in progesterone products equally reliably. We therefore i) tested the correlation between progesterone product concentrations measured on the same samples using the two assays; and ii) tested whether pre-partum falls in concentration could be detected with the same degree of statistical certainty by the two assays.

MATERIALS AND METHODS

Data of serum progesterone measurements of blood samples collected during the final weeks of pregnancy of nine pregnancies in four elephants at the study collection were reviewed. The average time of halving of progesterone product levels was calculated using the data of seven of these pregnancies. Serum samples taken during the same time frame and stored at -20°C were available for two pregnancies of two different elephants. Fresh serum samples were collected during a tenth pregnancy at the collection.

Commented [MR1]: How were these measurements made? All at the same lab as below?

I wonder if it would clearer to say simply that fresh samples from 10 pregnancies were analysed at the lab, and we were able to reanalyse samples from 3 of these at POC, from frozen serum in two cases and from fresh in one case.

Eighty-three samples had been submitted at the time of sampling to a reference laboratory (Nationwide Specialist Laboratories, London Rd, Pampisford, Cambridge CB22 3FJ, UK) to obtain progesterone measurements using a radioimmune assay[12] (IM1188, Backman Coulter®, Life Sciences Division Headquarters, 5350 Lakeview Parkway S Drive, Indianapolis, IN 46268, United States), with known cross-reactivity with 5α-pregnane. Of these, 73 duplicates were available for this study. In addition, 19 previously untested samples were included. In total, 92 samples were analysed: 81 archived frozen (-20ºC) samples of two separate pregnancies and 11 fresh samples of a third pregnancy (stored at 5°C and submitted to AgPlus Diagnostics Ltd and the external laboratory within 48 hours).

The Advantage Laboratory System® (AgPlus Diagnostics Ltd, The Exchange, Colworth Science Park, Sharnbrook, Bedford, UK) using canine progesterone materials and processes was used. This research and development system allowed for off line sample preparation and repeat measurements off a single device. Every stage was compliant with the ISO 9001 standard and ISO13485 for medical devices. The canine progesterone assay has been designed and developed to detect 4-pregnen-3,20-dione. The detection has been verified in canine serum and plasma samples against predicate clinical analyser data.

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The immunoassay system employs silver nanoparticles as an electrochemical label and magnetic particles as the solid phase. Antibodies/antigens specific to the target molecule are immobilised on the silver nanoparticles and a second antibody/antigen target onto a magnetic particle. After standard immunoassay and wash processes, the silver label is quantified using Anodic Stripping Voltammetry (ASV) and the signal generated is directly proportional to the amount of the target molecule in the sample. Frozen and fresh serum samples were brought up to room temperature (20ºC).

The canine assay is a competitive assay, in which free progesterone competes with progesterone analogues immobilised onto magnetic particles for available anti-progesterone antibody silver nanoparticle conjugates. As the levels of free progesterone increase, the resulting signal from the silver nanoparticles decreases. Canine serum samples perform optimally as a 1-in-21 dilution in PBS, however in initial testing in this study with elephant serum demonstrated a dilution factor of 1-in-8 was optimal, and the methodology was adapted accordingly.

Twenty µl of serum was mixed with 300 µl of dilution buffer (AgPlus Dilution Buffer, Phosphate Buffered Saline (PBS)). Of this mix, 150 µl was mixed with 15 µl of magnetic conjugate (AgPlus Progesterone Magnetic Conjugate, magnetic particles were coated with 4-pregnen-3,20-dione) and 15 µl of silver conjugate (AgPlus Progesterone Silver Conjugate, coated with a 4-pregnen-3,20-dione specific monoclonal antibody) and incubated at room temperature for three minutes. Using a magnetic rack, the conjugate was captured to remove supernatant and any unbound conjugate. The complex was re-suspended in 300 µl washing buffer (50mM iodoacetamide) and vortex mixed. Using a magnetic rack, the conjugate was captured again in order to remove the supernatant. The conjugate was subsequently re-suspended in 150 µl reaction buffer (1M ammonium thiocyanate). Tested samples were run in triplicate: three electrodes (Advantage laboratory Research & Development electrodes) were placed in a reader (Advantage Laboratory 3-channel Research & Development Reader) and 48 µl of the conjugate suspension was placed onto each electrode. Bespoke software subsequently recorded the signal. The raw signal values in nanocoulombs (nC) for all of the elephant samples were converted to ng/ml by reading the values against a standard elephant progesterone curve.

Raw results from the AgPlus reader testing (expressed in nanoCoulomb, nC) were correlated to the previously measured external laboratory progesterone results (nmol/litre). A

Commented [RV13]: Andrew, does this highlight the adaptation of the method between canine and elephant sufficiently?

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Commented [FM5]: Marcus/Andrew: The question is, are we going to do this paper in nmol/L or in ng/mL? As it's an American journal, I've changed the graphs to ng/mL, but can easily change it back. I'm happy to do the whole paper in nmol/L if that is easier/quicker. Please comment

Commented [MR6R5]: I have no view on this. Let me know and I'm happy re-plot the graphs as necessary

progesterone ‘calibration curve’ was generated to provide correlation data expressed in nmol/litre.

In order to identify anomalously low progesterone product concentrations, they were assessed against a normal distribution defined by the previously observed observations. Because there was often a clear trend in concentration over time, distributions were characterised with a linear model fit of concentration against time pre-partum, enabling prediction of the mean expected concentration at the time of subsequent measurements, while standard deviation is given by the root mean squared residuals of the linear model. Defining a normal distribution with this expected mean and standard deviation, the one-tailed probability of observing the observed concentration or lower can then be calculated, with lower probabilities indicating more strongly anomalous concentrations. Analysis was performed in R version 4.0.4 (R Core Team 2021).

Commented [MR7]: FULL REFERENCE
R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

RESULTS

For seven out of the nine previous pregnancies enough data were available from laboratory analyses to identify the sharp decline in blood progesterone products to half its pre-parturient level, on average 4.5 days prior to parturition (range 3-6 days, N=7), reaching undetectable levels 12-96h prior to delivery in all cases. The focus of the statistical analysis comparing laboratory with POC measurements was therefore on the last five days of pregnancy.

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The results of 5α-pregnane (radio-immune assay, external reference laboratory) and of 4-pregnen-3,20-dione (POC, AgPlus experimental reader) for Pregnancy 1, 2 and 3 are depicted in Figure 1. Patterns of change in progesterone product concentrations varied over time, between pregnancies and between methods. Concentrations of 5α-pregnane and 4-pregnen-3,20-dione were poorly correlated between the radio-immune assay and the POC, particularly when samples had been stored frozen (Pregnancies 1 and 2) (Figure 2).

Despite the lack of general correlation between results from the different assays, in both methods there was a substantial drop in progesterone product concentrations measured within five days of birth to levels well outside the 95% confidence limits for the distributions of concentrations seen prior to that (Fig. 2). Looking in more detail at statistical confidence in these pre-partum measurements as outliers, progesterone product concentrations near parturition were consistently more extreme than the lower 0.0001% of the previously observed distributions in all three pregnancies and regardless of the assay used, with significant drops detectable from either three or four days before birth (Figure 3). This indicates that both laboratory and point of care measurements can both accurately predict parturition a few days ahead.

Commented [MR9]: Hopefully this clarifies why the these confidence regions are shown in the fig, dealing with the reviewer's question on this.

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DISCUSSION

In this study, an adapted canine progesterone assay, which can be incorporated in a benchtop POC analyser, was able to detect the sudden decrease of blood progesterone products in Asian elephants and can therefore be used as a cost-effective tool for parturition prediction in this species.

Measurements show considerable variation, and substantial drops in apparent concentration were sometimes observed well before parturition (for example as measured at POC at Day -16 in Pregnancy 1, Figure 3), distinguished by a rapid return to previous levels, whereas repeat measurements just before parturition consistently showed continued decline, which could be for example due to sample storage, error or technical artefact. This suggests that a single low measurement should be followed by at least one further sample to confirm a

downward trend for accurate diagnosis of impending parturition. For Pregnancy 3, only 10 samples were available to establish an average level before the pre-parturition drop was detected, suggesting that the method can work reliably on smaller sample sizes.

The poor correlation between the POC output and the initial measurements obtained from the RIA may be due to the majority of the samples (88%) used for this study having been stored frozen for up to three years. Although serum progesterone concentrations should not be affected by frozen storage[14], in this study a number of samples stored over 2.5 years recorded a higher progesterone level on the POC analyser than they did in the laboratory at the time of sampling. Given the more concentrated nature of the elephant samples used in the assays compared to the canine assay, the freeze/thaw of samples frozen prior to this study would not have an impact on the progesterone but could have a larger impact on the serum itself, which could have a detrimental effect on the assay performance as observed in this study compared to freshly drawn samples. Refinement of the methodology, especially increasing the number of elephants studied plus using fresh serum samples, plus temporal tandem RIA analysis, would be needed to validate the POC for e.g. pregnancy diagnosis.

The analyte immobilised onto the magnetic beads for the canine assay is 4-pregnen-3,20-dione with an antibody specific to this form immobilised onto the silver nanoparticles. Whilst this study would suggest some cross reactivity of this antibody towards progesterone products more commonly found in elephants such as 5α-pregnane, the level of cross reactivity is unknown. Similarly, any forms of 4-pregnen-3,20-dione in samples will likely have a higher affinity towards the antibody used in this study and may impact results. Further investigation into the cross reactivity of this antibody and potential antibodies more specific to elephant progesterone products should be explored in future work.

The main drawback of this method of parturition prediction using a POC progesterone analyser is the need for serial blood samples. Non-invasive sampling methods may be

preferred in some collections. Progesterone can also be measured in the urine of elephants[10] and in saliva in human[13,8]. Both could potentially be a focus for future research.

CONCLUSION

As pregnancy length in elephants is highly variable, and neonatal deaths still occur with some regularity, reliable parturition prediction in captive Asian elephants is invaluable for ensuring staff are on hand to assist if required. Where serial blood samples of elephants can be obtained, objective parturition prediction is possible using the POC analyser used in this study, and can direct the need for ultrasonographic evaluation of the cervix when parturition does not follow a progesterone drop in the expected interval.

ACKNOWLEDGEMENTS

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References

1. Boes KM, Sink CA, Camus MS, Werre SR. Evaluation of an in-clinic dry chemistry analyzer for canine, equine, and feline plasma samples. J Vet Diagn Invest. 2018;30(6):902-910.

2. Brown JL, Lehnhardt J. Serum and urinary hormones during pregnancy and the peri- and postpartum period in an Asian elephant (*Elephas maximus*). Zoo Biology. 1995;14:555-564.

3. Carden M, Schmitt D, Tomasi T, Bradford J, Moll D, Brown JL. Utility of serum progesterone and prolactin analysis for assessing reproductive status in the Asian elephant (*Elephas maximus*). Anim Reprod Sci. 1998;53:133-142.

4. Doyle C, York B, Whitely A. A survey of Asian elephants births from 1962-1998. Journal of the Elephant Managers Association. 1999;10:146-148.

5. Elmeshreghi TN, Grubb TL, Greene SA, Ragle CA, Wardrop JA. Comparison of Enterprise Point-of-Care and Nova Biomedical Critical Care Xpress analyzers for determination of arterial pH, blood gas, and electrolyte values in canine and equine blood. Vet Clin Pathol. 2018;47(3):415-424.

6. Hermes R, Saragusty J, Schaftenaar W, Goritz F, Schmitt DL, Hildebrandt TB. Obstetrics in elephants. Theriogenology. 2008;70(2):131-144.

7. Hodges JK, Heistermann M, Beard A, van Aarde RJ. Concentrations of progesterone and the 5 alpha-reduced progestins, 5 alpha-pregnane-3,20-dione and 3 alpha-hydroxy-5 alpha-pregnan-20-one, in luteal tissue and circulating blood and their relationship to luteal function in the African elephant, *Loxodonta africana*. Biol Reprod. 1997;56(3):640-646.

8. Maged AM, Mohesen M, Elhalwagy A, Abdelhafiz A. Salivary progesterone and cervical length measurement as predictors of spontaneous preterm birth. J Matern Fetal Neonatal Med. 2015;28(10):1147-1151.

9. Niemuller C, Gray C, Cummings E, Liptrap RM. Plasma concentrations of immunoreactive relaxin activity and progesterone in a pregnant Asian elephant (*Elephas maximus*). Anim Reprod Sci. 1998;53:119-131.

10. Niemuller CA, Shaw HJ, Hodges JK. Noninvasive monitoring of ovarian function in Asian elephants (*Elephas maximus*) by measurement of urinary 5ß-pregnanetriol. J Reprod Fertil. 1993;99:243-251.

11. Niemuller CA, Shaw HJ, Hodges JK. Pregnancy determination in the Asian elephant (Elephas maximus): A change in plasma progesterone to 17α Hydroxyprogesterone ratio. Zoo Biology. 1997;16:415-426.

12. Olsen JH, BChen CL, Boules MM, Morris LS, Coville BR. Determination of reproductive cyclicity and pregnancy in Asian elephants (*Elephas maximus*) by rapid radioimmunoassay of serum progesterone. Journal of Zoo and Wildlife Medicine. 1994;25:349-354.

13. Priya B, Mustafa MD, Guleria K, Vaid NB, Banerjee BD, Ahmed RS. Salivary progesterone as a biochemical marker to predict early preterm birth in asymptomatic high-risk women. BJOG. 2013;120(8):1003-1011.

14. Reyna R, Traynor KD, Hines G, Boots LR, Azziz R. Repeated freezing and thawing does not generally alter assay results for several commonly studied reproductive hormones. Fertil Steril. 2001;76(4):823-825.

15. Schwarzenberger F, Strauss G, Hoppen H-O, Schaftenaar W, Dieleman SJ, Zenker W, Pagan O. Evaluation of progesterone and 20-oxoprogestagens in teh plasma of Asian (Elephas maximus) and African (Loxodonta africana) elephants. Zoo Biology. 1997;16:403-413.

16. Slovis NM, Browne N, Bozorgmanesh R. Point-of-Care Diagnostics in Equine Practice. Vet Clin North Am Equine Pract. 2020;36(1):161-171.

17. Waldmann A, Raud A. Comparison of a lateral flow milk progesterone test with enzyme immunoassay as an aid for reproductive status determination in cows. Vet Rec. 2016;178(11):260.

Figure 1a-c: Readings of 5α-pregnane (radio-immune assay, external reference laboratory) and of 4 pregnen-3,20-dione (POC, AgPlus experimental reader) for all three pregnancies in ng/mL, in graph format.

Figure 2. Correlations between progesterone product concentrations (ng/ml) measured either in the laboratory (lab) or at point of care (POC) during the late stage of three Asian elephant pregnancies. Open points are within five days of parturition, filled points are earlier. Shaded ellipses are bivariate normal 95% confidence regions. The grey line indicates equality. Pearson correlation statistics for pregnancies 1: r = -0.15, p = 0.38; 2: r = 0.44, p = 0.04; 3: r = 0.28; p = 0.46.

Figure 3. Normal distribution probabilities of observed progesterone concentrations within 20 days of parturition, based on prior observations, for three Asian elephant pregnancies (rows), measured either in the laboratory (lab column) or at point of care (POC). Horizontal grey lines indicate the 0.001 probability threshold. Vertical grey lines indicate the threshold between four and five days pre-partum.

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Note that the figure numbers have gone one up because the AE requested the data in graph format, so the raw data turned into Figure 1.

why do figures 1 and 2 use 5 days prior to parturition, and figure 3 uses 4 days prior?

what is the relevance of the bivariate normal 95% confidence region? Is it possible to use the same axis scale for each axis (make the intersection zero for both)?

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