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Laboratory and Clinical Evaluation of a Feia Method for Canine Serum Progesterone Assay

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Contents
The evaluation of progesterone (P4) concentration is a valuable tool in assessing physiological reproductive events and reproductive disorders in bitches. A reliable and rapid (preferable, point of care) determination of P4 is advisable in most cases. Aims of this study were to evaluate a fluorescence enzyme immunoassay (FEIA) for canine serum P4 concentration by (i) the agreement with liquid chromatography–tandem mass spectrometry (LC/MS/MS), (ii) the association with vaginal cytology and (iii) the accuracy in the prediction of the parturition date calculated from the estimated day of ovulation. Serum samples were collected from client-owned bitches presented between 2011 and 2014 for the evaluation of their oestrous cycle, pregnancy or reproductive disorders. The agreement between FEIA and LC/MS/MS, evaluated on 19 samples, was statistically significant (R^2 = 95.7%, $p < 0.001$), although FEIA showed significantly higher values than LC/MS/MS ($p < 0.05$). In the different phases of oestrous cycle, as determined by vaginal cytology, P4 concentrations (by FEIA) were statistically different ($p < 0.05$): anoestrus (n = 7) 0.38 ± 0.14 ng/ml, proestrus (n = 14) 1.04 ± 0.67 ng/ml and oestrus (n = 72) 6.8 ± 7.26 ng/ml. Mean pregnancy length from the estimated day of ovulation was 62.9 ± 1.8 days. In 13 of 22 (59.1%), 19 of 22 (86.3%) and 21 of 22 (95.5%) bitches pregnancy lasted 63 ± 1, 63 ± 2 and 63 ± 3 days, respectively. Three pregnancies were outside the 61–65 days range (60, 60 and 67 days). In conclusion, the FEIA method employed can be considered reliable and, in association with vaginal cytology, effective in evaluating the canine oestrous cycle.

Introduction

In Italy, more than 130 000 puppies are registered at the kennel club (Ente Nazionale della Cinofilia Italiana, the national organization responsible to issue dogs' pedigrees) every year, and hundreds of working competition (hunting, utility, agility, sheepdog, coursing, etc.) and dog shows are organized. Dog breeding is an economically important activity, as it drives an economic engine including the sale of puppies, pet food and accessories, animal health and veterinary services, pet insurances, and dog activities (FECAVA 2007). It has been estimated that companion animals in the United States move nearly forty billion dollars per year for food, accessories, veterinary care and other support services and products (Zawistowski 2008). Canine events are today organized in most world's countries and successful show or working dogs together with their owners travel the whole year around, adding journey and housing expenses. The value of these dogs can be very high and owners require increasingly sophisticated veterinary evaluations and treatments, including specialists in reproduction, for the examinations of both males and females.

The evaluation of progesterone (P4) concentration is a valuable tool in assessing reproductive events in bitches. Stage of oestrous cycle and ovulation time can be determined by the combination of vaginal cytology and serum P4 concentration. Ovulation occurs when a bitch in cytological oestrus reaches 4–8 ng/ml of P4, and best breeding days for natural mating are considered those in the range from 1 to 4 days post-ovulation, while for frozen semen, the window is narrower and closer to day 4 (Badinand et al. 1993; Steckler et al. 2013). Pregnancy length from the day of ovulation has been described to range 62–64 days (Concannon et al. 2001; Tsutsui et al. 2006; Fontbonne 2008), 61–65 days (Okkens et al. 2001; Elits et al. 2005) or even 58–67 (Mir et al. 2011). Progesterone levels decrease significantly (<1–2 ng/ml) 24 h before parturition (Concannon et al. 1978; Chakraborty 1987; Hoffmann et al. 1994; England and Verstegen 1996; Veronesi et al. 2002). Progesterone can thus be employed both to predict with enough accuracy the date of parturition and to evaluate whether natural parturition is impending or a planned Caesarean section (C-S) should be performed (Smith 2007), which is particularly important due to the late pulmonary maturation of canine foetuses (Sipriani et al. 2009). Moreover, knowledge of circulating progesterone concentration is useful for the diagnosis and treatment of reproductive disorders such as pyometra, abnormalities of oestrous cycle, diseases of pregnancy and ovarian remnant syndrome (e.g. Meyers-Wallen 2007; Sontas et al. 2014; Krauss and Krauss 2015).

A reliable and rapid (preferable, point of care) determination of P4 is often advisable, that is when the time of ovulation needs to be determined for a mating or an artificial insemination, or when a decision has to be made whether a C-S should be performed or a treatment initiated. Several laboratory methods have been employed for the quantification of P4. Radioimmunoassay (RIA) is today still considered the reference method; however, its use is limited due to practical problems, such as those linked to the use of gamma-radioactive isotopes. For this reason, methods more often used today in canine practice and research are immunoassays, both by chemiluminescence (CLIA, e.g. Forsberg et al. 1993; Kutzler et al. 2003; Chapwanya et al. 2008; Mir et al. 2011) and by fluorescence enzyme

immunoassay (FEIA, Brugger et al. 2011). Quantitative ELISA has also been validated and used in clinical studies (e.g. Ververidis et al. 2002; Moxon et al. 2010; Seki et al. 2010). For clinical practice, ELISA kits are available, although they resulted to have varying accuracies when tested (van Klaveren et al. 2001; Moxon et al. 2010). More recently, mass spectrometry has been introduced for the evaluation of numerous molecules including steroids, and it is considered today's gold standard in humans (Tai et al. 2006; Soldin and Soldin 2009; Field 2013). In veterinary medicine, its use for P4 determination has been validated in cattle, and it has also been employed for the quantification of six sex hormones (pregnenolone, progesterone, oestrone, testosterone, androstenedione and dehydroepiandrosterone) in bovine milk (Regal et al. 2009, 2012; Fernandes et al. 2011). In the recent years, a FEIA assay (MiniVidas, BioMerieux, France) has been frequently used in canine research and practice (Grellet et al. 2012; Alonge et al. 2013; Krachudel et al. 2013). A new instrument (AIA®-360, TOSOH Corp. Japan), employing a different FEIA method, was recently evaluated and resulted to be accurate and to have a high agreement with CLIA (Monino et al. 2012). This new FEIA instrument, however, was not yet tested in the clinical environment.

Aims of this study were to evaluate the following: (i) the agreement between FEIA and liquid chromatography–tandem mass spectrometry (LC/MS/MS) to assay canine serum P4 concentration, (ii) the association between vaginal cytology and P4 concentration evaluated by the FEIA assay (iii) the accuracy of the prediction of the parturition date calculated from the day of ovulation as estimated by FEIA assay.

Materials and Methods

This study was conducted at the Veterinary Teaching Hospital of the Department of Veterinary Sciences, University of Pisa, Italy. Samples were collected from client-owned bitches presented between 2011 and 2014 for the evaluation of their oestrous cycle, pregnancy or reproductive disease.

The owners for the use of the samples signed an informed consent.

Sampling

Blood samples were collected from the cephalic or jugular vein, placed in 4-ml serum sample tubes and allowed to clot for 30 min. Serum was separated by centrifugation (1300 *g* for 10 min) and submitted to FEIA assay within 2 h from collection. Aliquots for LS-MS/MS were kept frozen (−18°C) until evaluation.

Progesterone evaluation

The instrument used for FEIA evaluation was AIA®360 (TOSOH Corp., Minato-ku, Tokyo, Japan), and the kit employed was ST AIA PACK PROG with the fluorogenic substrate 4MUP (TOSOH Corp.). This analyser uses a competitive fluorescent enzyme immunoassay, which is performed entirely within small, single-use test cup containing all necessary reagents. The analyte present in the sample (0.5 ml) competes with enzyme-labelled hormone for a limited number of binding sites on hormone-specific antibodies immobilized on magnetic beads. Reported intra- and interassay CV% for canine serum progesterone measurements with this method were 2.01–6.67% and 5.09–15.88%, respectively, depending on progesterone concentration (Monino et al. 2012). In a subset of the samples included in this study (n = 16), measurements were repeated twice and intra-assay CV% was 3.8%.

The LC-MS/MS was performed at Laboratorio Privato di Analisi Veterinarie 'San Marco', Padova, Italy, using a nanoUPLC/XevoQtof (Waters Corp., Milford, MA, USA). The samples (0.1 ml) were analysed in ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS-MS) operating in multiple reactions monitoring (MRM mode) in electrospray ionization (ESI) positive (Melechco Carvalho 2012; Methlie et al. 2013). The analyte was directly determined without the need of derivatization. The UPLC/MS-MS variables were optimized and calibrated in a wide concentration range (0.01–100 ng/ml). The recovery of progesterone was >82% with CV of <5%. The within-day and between-day precision assays were 2.82–5.81% and 3.29–9.62%, respectively. The accuracy and precision were evaluated from replicate analyses of QC sample at three different final concentrations.

Experimental design

Agreement between FEIA and LS-MS/MS

To evaluate the agreement of the two methods, 19 serum samples at varying P4 concentrations (range 0.7–34.2 ng/ml, as determined by FEIA) were collected from bitches presented for staging of the cycle. In this study, samples were evaluated both by FEIA and LC/MS/MS.

Association between vaginal cytology and P4 concentration evaluated by the FEIA assay

Fifty-five bitches presented for the evaluation of the oestrous cycle were included. In most cases, the reason for consulting was the management of mating or artificial insemination while in some occasions was to investigate cycle abnormalities (primary anoestrus, frequent or prolonged heats).

On these bitches, a total of 93 samples of smears for vaginal cytology combined with P4 evaluation by FEIA assay were performed. For vaginal cytology evaluation, smears were stained with Diff-Quik® and classified based upon epithelial cells morphology and the presence of neutrophils and erythrocytes (Roszel 1977).

Accuracy of the prediction of the parturition date calculated from the day of ovulation estimated by the FEIA assay

Pregnancy length was evaluated in 22 bitches included in the previous group. Inclusion criteria were as follows: (i) at least one serum P4 determination between 2 and 13 ng/ml, interpreted as follows: (a) 2.0–3.0 ng/ml as 2 days before ovulation, (b) 3.01–4.0 as 1 day before ovulation, (c) 4.01–8.0 as the day of ovulation, (d) 8.01–13.0 as 1 day post-ovulation; (ii) pregnancy without complications; (iii) natural delivery or C-S due to dystocia (elective C-S were not included). A pregnancy length equal to 63 ± 2 days from estimated ovulation was considered a positive result (adequate accuracy of the prediction of parturition date).

Statistical analyses

The agreement between FEIA and LC/MS/MS was evaluated by linear regression analysis and by Pearson's correlation, while the difference between the two tests was evaluated by paired *t*-test. The Kruskal–Wallis test, followed by the Mann–Whitney *U*-test for *post hoc* pairwise comparison, was used to compare progesterone concentrations in the different stages of oestrous cycle. Data were presented as means ± standard deviations. Differences were considered statistically significant when $p < 0.05$. Analyses were performed using the statistical package Minitab 16.1 (Minitab Inc., State College, USA).

Results

Agreement between FEIA and LS-MS/MS

The agreement between FEIA and LC/MS/MS was statistically significant ($R^2 = 95.7\%$, $p < 0.001$, Figure 1), and the relation between the two methods is described by the following regression equation: P4 ng/ml FEIA = −0.2761 + 1.274*P4 ng/ml LC/MS/MS. Pearson's correlation was also significant ($r = 0.979$; $p < 0.001$). Despite the agreement between the two methods, however, FEIA showed significantly higher values than LC/MS/MS ($p < 0.05$).

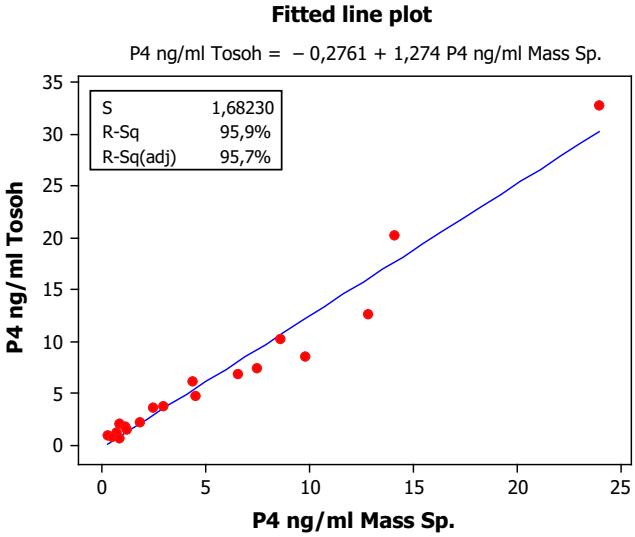

Fig. 1. Fitted line plot and regression analysis describing the agreement between the two tested methods, FEIA and LC/MS/MS

Association between vaginal cytology and P4 concentration evaluated by the FEIA assay

In the different phases of oestrous cycle, as determined by vaginal cytology, the following P4 concentrations were observed: anoestrus (n = 7) 0.38 ± 0.14 ng/ml (range: 0.25–0.68), proestrus (n = 14) 1.04 ± 0.67 ng/ml (range: 0.31–2.56), and oestrus (n = 72) 6.8 ± 7.26 ng/ml (range: 0.37–32.9). Values obtained in the three phases were statistically different ($p < 0.001$, Fig. 2), lower values were observed in anoestrus compared to proestrus ($p < 0.01$) or oestrus ($p < 0.001$) and in proestrus compared to oestrus ($p < 0.001$).

Accuracy of the prediction of the parturition date calculated from the day of ovulation estimated by the FEIA assay

Mean pregnancy length was 62.9 ± 1.8 days (range 60–67). In 13 of 22 (59.1%), 19 of 22 (86.3%) and 21 of 22 (95.5%) bitches, the pregnancy lasted 63 ± 1, 63 ± 2 and 63 ± 3 days, respectively. The three pregnancies outside the 61–65 days range lasted 60, 60 and 67 days (Fig. 3).

Discussion

The FEIA method employed in this study was previously validated for canine P4 evaluation by Monino et al. (2012) which showed a very high level of agreement between FEIA and CLIA ($R^2 = 0.978$) and low

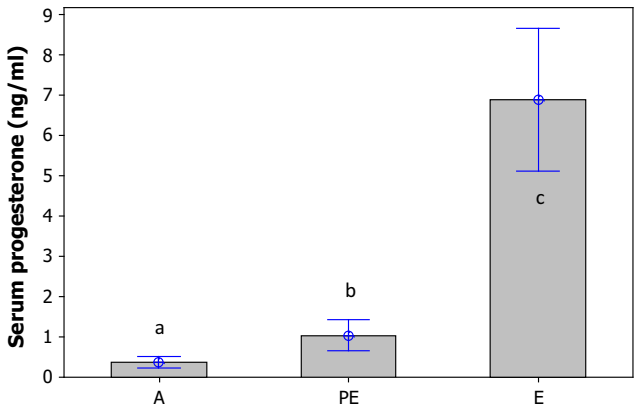

Fig. 2. Serum progesterone concentrations (mean and 95% CI for the mean, FEIA assay) in the different phases of oestrous cycle in bitches, as determined by vaginal cytology (A: anestrus, PE: proestrus and E: oestrus; a≠b≠c, $p < 0.01$)

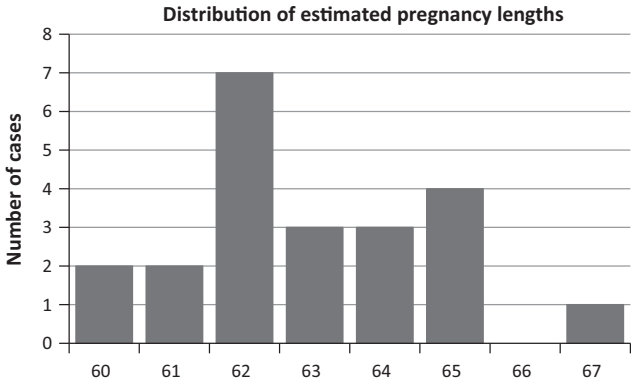

Fig. 3. Distribution of pregnancy lengths from the day of estimated ovulation in 22 bitches

intra-assay and interassay coefficients of variation. The agreement of FEIA with LS/MS/MS confirmed the reliability of the FEIA method. In humans, the comparison of the same two methods employed in the present study yielded a higher correlation ($r = 0.995$) but a lower slope ($R^2 = 0.75$, Patton et al. 2014) than the one observed for dogs. This might be due to a species difference or to a different distribution of samples P4 concentrations. From our data set, significantly higher P4 values are obtained with FEIA, compared to LS/MS/MS, and the possible implications of the different results should be taken into account in the clinical situation. In the present study, however, differences between the two methods were small both up to 8 ng/ml (average mean difference: 0.58 ± 0.52 ng/ml, range −0.1 to 1.18) and between 8 and 13 ng/ml (average mean difference: 0.10 ± 1.43 ng/ml, range −1.20 to 1.64). The FEIA method should thus be adequate to monitor reproductive events such as oestrous cycle or pregnancy. Due to the clinical environment, FEIA samples were evaluated fresh, while those submitted to LS/MS/MS were kept at −18°C until evaluation. However, the different conditions between sampling and evaluation were probably not the reason for this discrepancy, as subjecting samples to freezing and thawing and even at several freeze–thaw cycles was shown not to affect P4 concentration (Tahir et al. 2013).

The use of vaginal cytology in the management of oestrous cycle is advisable as it allows to define the stage of oestrous cycle, to interpret better P4 analysis results and in some occasion aids to diagnose reproductive tract disorders. However, vaginal cytology alone is not adequate when ovulation has to be precisely determined to plan a single natural mating or artificial insemination. It is clear from our data that, although oestrous cycle stages assessed by vaginal cytology differed significantly in P4 concentration, oestrus was sometimes diagnosed very early with this method compared to the time when ovulation would have occurred as determined by progesterone concentration (2–6 days earlier). This might be due both to the subjective nature of this evaluation and to the different patterns in vaginal epithelium keratinization occurring in different bitches (England 1992), and underlines the importance of the use of progesterone analyses when planning a mating. Progesterone concentration is also an important examination when dealing with abnormal oestrous cycles. Four samples collected from the same bitch showed low progesterone concentrations despite oestrous vaginal smears: this bitch was lately diagnosed as having a follicular cyst.

In the literature, pregnancy length has been described from the day of mating, luteinizing hormone (LH) surge or ovulation. From mating, prediction of parturition is not accurate, as pregnancy has been shown to last from 57 to 72 days (Concannon et al. 1978). From LH peak, on the contrary, parturition occurs after 65 ± 1 days (Concannon et al. 1978). However, laboratory tests to evaluate canine LH are scarce and rather expensive, and LH surge is short and needs daily evaluations to be detected. For this reason, some authors have defined the day of LH surge, using P4 assays, as the day when P4 first reaches 1.5 ng/ml and increases thereafter (Kutzler et al. 2003). The accuracy in the prediction of parturition date with the protocol employed in the present study is comparable to that observed calculating pregnancy length from the first day when P4 concentration reached 1.5 ng/ml (65 ± 2 days in 90% of cases, Kutzler et al. 2003) or from the day of estimated ovulation (63.1 ± 2.1 days, Mir et al. 2011). Pregnancy length was 63 ± 2 days, and 86.3% of the bitches gave birth to the puppies between 61 and 65 days. However, the overall range was 60–67 days, similar to what was previously described by Mir et al. 2011. Several factors have been evaluated for their influence on pregnancy length: size, breed, weight, age, parity and litter size. While size, weight and breed might have an effect, age and parity could not be associated with pregnancy length (Okkens et al. 2001; Seki et al. 2010; Mir et al. 2011). Litter size had a significant effect in some studies, where small litters (1–2 pups) originated from longer pregnancies (Mir et al. 2011), but not in others (Kutzler et al. 2003; Seki et al. 2010).

In conclusion, the FEIA method employed can be considered reliable and, in association with vaginal cytology, effective in evaluating the canine oestrous cycle.

Author contributions

AR, IV and SM performed the sample collection from clinical cases, while AG and GL were responsible for the laboratory analyses. AR designed the study. AR and SM performed the statistical analyses. All authors interpreted the results. AR and SM drafted the manuscript. All authors contributed to and approved the submitted version of the article.

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