Comparison of Three Different Analyzers to Measure Canine Serum Progesterone

by
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AN ABSTRACT OF THE THESIS OF

Trevor Hisanaga for the degree of <u>Honors Baccalaureate of Science in Biology</u> presented on May 21, 2021. Title: <u>Comparison of three different analyzers to measure canine serum progesterone</u>.

Abstract approved:_____

Michelle Kutzler

The canine estrous cycle is unique compared to other domestic animal species. During estrus, serum progesterone concentrations ([P4]) rise two days prior to ovulation. In fact, the luteinization of pre-ovulatory follicles resulting in this initial increase in [P4] (1.5-2.5 ng/ml) cannot be temporally dissociated from the onset of the surge in luteinizing hormone. For this reason, measuring progesterone from daily blood samples is commonly used to determine the optimal breeding day in female dogs. In addition, the fall in [P4] (<2 ng/ml) prior to parturition can be used for the purposes of determining the timing of an elective C-section in dogs.

There are several methods veterinarians can use to measure [P4] but directly comparing results between assays without formulaic adjustments often yields unreliable results. The objective of this research was to compare [P4] measured on three different

veterinary analyzers (enzyme linked fluorescent assay (**ELFA**), colorimetric immunoassay (**CIA**), and chemiluminescent immunoassay (**CLIA**)).

It was hypothesized that irrespective of analyzer used, the [P4] measurement would be reliable for determining timing for breeding or C-section. Venous blood samples (n=116) were collected from privately-owned female dogs (n=44) at the Waipahu Waikele Pet Hospital in Honolulu, Hawaii. Dogs were fasted 6 to 8 hours prior to each blood collection and blood samples were collected into tubes containing a clot activator but not serum separating gel. Blood samples were allowed to clot for 30 to 60 minutes and then were centrifuged for five minutes at 3,500 rpm to allow for serum removal. In accordance with the manufacturer's instructions, [P4] was determined from three analyzers.

Data were managed in Google Sheets and analyzed using R. Using simple linear regression, the coefficients of determination (R^2) between CIA and ELFA, CIA and CLIA, and CLIA and ELFA was 0.88, 0.914, and 0.957, respectively. The simple linear regression was also used to determine the regression equation, which was CLIA = 0.536*CIA+0.688, CLIA = 0.417*ELFA+0.321, and CIA = -0.45*ELFA+0.73. Using a Passing-Bablok regression, the Pearson correlation coefficients were 0.938, 0.956, and 0.978, respectively. However, analysis of the residuals showed an increase in the spread as the [P4] increased. The results from this study show that comparison of [P4] between the analyzers is accurate at lower values (<5 ng/mL) but the variability in [P4] increases as the value increases.

Key Words: Progesterone, Canine Reproduction, Immunoassay

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Trevor Hisanaga, Author

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Introduction:

<u>Overview of Canine Reproduction</u>

   Canine reproduction is a growing field within veterinary medicine, with an estimated annual shortage of two million pet dogs in the United States[1]. Additionally, there is an especially high demand for purebred and "designer" dogs[2]. In order to maximize the chances of conception, breeding should be performed at the optimal time during estrus. Determining the optimal time for breeding is challenging because of the unique features of the canine estrous cycle.

   Except for the Basenji, domestic dogs are nonseasonal, meaning that breeding can occur during any time of the year. Dogs are also monoestrous, meaning that they experience a period of anestrus with each estrous cycle[3]. The canine estrous cycle is split into four stages: proestrus, estrus, diestrus, and anestrus[3]. Estrus (the period of sexual receptivity) lasts for an average of nine days (range: 3-21 days)[3,4]. Estrus is also the stage when ovulation occurs[3]. Ovulation is caused by an abrupt surge in luteinizing hormone (LH) at the end of proestrus and it takes place 48-60 hours after the onset of the LH surge (day 0)[3]. However, canine oocytes are not fertilizable at the time of ovulation because they have not completed Meiosis I. Primary canine oocytes mature into secondary oocytes (capable of being fertilized) not sooner than 54 hours after ovulation[5]. Canine secondary oocytes remain fertilizable for up to 108 hours after ovulation[6].

   The sperm of some male dogs can retain its fertilizing capacity within the reproductive tract of some female dogs for as short as 12 hours or as long as 10 days[7,8]. Additionally, the likelihood of conception from a single mating is at its maximum

between 0 and 5 days after the LH surge[3]. This variance makes it difficult to determine if a mating resulted in the presence of viable sperm during the period of time when canine secondary oocytes were still fertilizable. This variability in sperm longevity also makes it harder to accurately predict the date of parturition from the breeding date alone[9,10]. Fertility rapidly drops 7-8 days after the LH surge, in part due to cervical closure[3]. Understanding this is especially important with shipped or frozen semen because insemination at the incorrect time will not result in pregnancy and will therefore be costly to the dog owner[10,11].

Length of gestation can be predicted if the date of the LH surge is known (65 ±1 days). If the date of the LH surge is not known, apparent gestation length can range from 56-72 days from the first of a series of matings[4]. Placental mammalian parturition, which includes canines, can be divided into three stages, with stage 1 being preparation of the uterus and cervix for birth, stage 2 being active labor with delivery of the fetuses, and stage 3 being expulsion of the fetal membranes[12]. In canines, parturition usually lasts 4-18 hours, depending on parity, litter size, and the absence of complications[12]. Twenty-four hours prior to parturition, there is an increase in cortisol which is followed by a decrease in [P4][12]. Since progesterone has thermogenic effects, an abrupt decrease in [P4] results in an abrupt decrease in body temperature by 2ºF (1ºC) when compared to a previous morning/night[12-15]. If the date of the LH surge is not known, an elective C-section can be scheduled based upon this drop in body temperature. The abrupt decrease in [P4] will also induce an increase in serum prolactin concentrations[12]. Elevated prolactin concentrations initiate lactation and cause the pre-partum behaviors of inappetence, social withdrawal/defensiveness, and nesting (e.g., digging into bedding)[12].

During stage 1 of parturition, nesting behavior is intensified and panting and whining can be observed while the cervix is dilating and uterine contractions are initiated.

Timing Canine Insemination to Maximize Success

There are several methods used to determine the optimal time for breeding but methods measuring [P4] and LH concentrations are the most accurate[10,16]. As mentioned prior, serum LH concentrations surge 48-60 hours before ovulation[3,9]. However, the LH surge is brief, lasting for less than 48 hours[10]. To predict ovulation using LH measurement, samples would need to be collected daily, which may be impractical for most clinical settings[16,17]. The onset of the LH surge cannot be temporally disassociated with the initial rise in [P4] above 2 ng/ml [4,11,16]. The initial rise in [P4] can be detected when measured every other day, making this method less stressful on the dog and more affordable for the dog owner[16]. After the LH surge, the continued rise in [P4] (measured in nanograms per milliliter or ng/ml) is highly variable between dogs due to differences in body weight (e.g., total blood volume in circulation in milliliters) and the number of ovulations that cycle (e.g., amount of P4 produced in nanograms)[16].

Predicting Parturition for Scheduling an Elective C-section

In addition to determining the optimal time for breeding, serial measurements of [P4] are used to determine the timing of an elective C-section. The rationale for performing an elective C-section (as opposed to an emergency C-section) is that several breeds, especially brachycephalic breeds, have a high risk for dystocia[18]. The likelihood that all puppies would survive an emergency C-section is much lower than the likelihood

that they would survive an elective C-section[18]. In addition, bitches with abnormalities in the birth canal (e.g., vaginal stricture) may be unable to deliver without assistance[19]. As previously mentioned, the apparent gestation length can range from 56-72 days from the first of a series of matings[4]. However, 12-40 hours prior to birth of the first puppy, [P4] drops to below 1.5 ng/ml[20]. Since this decline in [P4] is consistent across all pregnant dogs, it serves as a reliable determinant of fetal readiness for delivery.

Assays Used to Measure Canine Serum Progesterone Concentrations

Serum progesterone concentrations ([P4]) can be measured using different methods. One such method is liquid chromatography mass spectrometry (**LC-MS**)[21]. This method uses liquid chromatography to separate out the substances in a sample, including progesterone, and then uses mass spectrometry to quantify the amount of each compound present in a sample[21]. While this method is accurate enough to be considered a gold standard, it isn't commonly used in clinical veterinary medicine because of its long run time. In cases where [P4] levels need to be assessed for emergency C-sections or unscheduled artificial inseminations, longer report times may result in complications for the practicing veterinarian.

Immunoassays are more commonly used to assess [P4]. Immunoassays use antibodies directed against a specific antigen (in this case P4), but each assay differs slightly in the method of detection[22]. Radioimmunoassays (**RIA**) measure gamma radiation emitted from radioactive iodide (^{121}I)- or tritium (^{3}H)-labeled to the antibody[23-25]. Although RIA is the gold standard, it is not available for clinical practice due to its use of radioactive materials, high price, long reporting times, and strict requirements for

professional operation[23-25]. A variety of non-radioactive enzyme-linked immunoassay methods are available for use in clinical practice including enzyme linked fluorescent assay (**ELFA**), colorimetric immunoassay (**CIA**), and chemiluminescence immunoassay (**CLIA**) (**Table 1**)[26-30]. Each assay is competitive, meaning that P4 in the sample competes with enzyme-tagged P4 to bind to antibodies attached to the walls of the well, resulting in a reading in which the amount of serum P4 in the sample is inversely proportional to the intensity of the luminescence (CLIA), fluorescence (ELFA) or coloration (CIA) produced[26-30]. It is worth noting that while the CLIA test examined in the current study used enzymes to produce its luminescence, non-enzymatic luminophore markers can also be used with CLIA[29,31].

Table 1: Analyzer information.

Name of Analyzer	Type of Analyzer
Mini Vidas	Enzyme linked fluorescent assay (**ELFA**)
Catalyst Progesterone	Colorimetric immunoassay (**CIA**)
Idexx Reference Labs (Immulite 2000 Progesterone)	Chemiluminescent immunoassay (**CLIA**)

Results can vary between these assays as well as with the same assay because of differences in lots of reagents and how each analyzer is calibrated. Calibration points use a series of known concentrations of progesterone and often varies between assays. If the

same sample was analyzed on two different analyzers, the results may be slightly different. In clinical practice, veterinarians may not have access to the same analyzer each day when determining the timing for insemination or elective C-section. Direct comparisons between each method are needed to determine how veterinarians can interpret these results when making clinical decisions. Previous studies have compared non-radioactive assays to RIA due to their status as the gold standard, but no previous studies directly compared other non-radioactive immunoassays[23,24,30,32].

Thesis Objective and Hypothesis

The objective of this thesis research was to compare [P4] analyzed concurrently on multiple assays. It was hypothesized that [P4] would correlate between assays.

Methods:

During routine reproductive procedures, trained assistants and certified technicians working at Waipahu Waikele Pet Hospital in Honolulu, HI, collected venous blood from client-owned female dogs (n=44; **Table 2**). Dogs were fasted 6 to 8 hours prior to blood collection. Also, the time-of-day blood was collected varied considerably but fell within the range of 7:30 am to 3 pm. The blood samples were collected to measure serum progesterone concentration ([P4]) for determining the optimal timing for artificial insemination or elective C-section. Depending upon the reproductive procedure, multiple serial blood samples were collected from the same dog. Each sample (n=116; **Table 2**) was placed into a red top Vacutainer® tube with clot activator but not serum separating gel. Samples were allowed to clot for 30 to 60 minutes and then were centrifuged for five minutes at 3,500 rpm.

Serum from each sample was transferred into a 6 mL white top Vacutainer® tube containing no additives and then processed on an CIA progesterone analyzer (**Figure 1A**). The standard procedure described by the manufacturer was used without any deviation[33]. The remainder of each serum sample was stored at -20° C for testing on additional analyzers – ELFA (**Figure 1B**) and CLIA reference laboratory analyzer. The standard procedure described by the manufacturer for the ELFA was used without any deviation[34]. For the samples analyzed by CLIA, a courier picked up each sample and delivered it to the reference laboratory for analysis. Due to limited sample volume, some samples were only tested using two methods.

Data were managed in Google Sheets and analyzed using R. Using the Passing-Bablok method, each machine was compared to the other two resulting in a total of three

comparisons (ELFA vs CIA, ELFA vs CLIA, and CIA vs CLIA). The Passing-Bablok method is a nonparametric method commonly used to compare two devices that should be giving the same measurement[35]. The regression equation and Pearson correlation coefficient (Pearson's r) were reported. The Pearson's r is a measure of correlation between the two assays[35]. Values over 0.9 were considered to be highly correlated[35]. Additionally, the Passing-Bablok regressions contained a line of identity (Figure 5). A line of identity is created by plotting y=x. This line represents how the data would look if the two devices gave a 1:1 correlation in data[36]. Regressions were analyzed with a residuals plot to ensure assumptions of linearity were met.

Table 2: Signalment of dogs used in the study (n=44).

ID	Age*	Breed	# of blood samples collected total		ID	Age*	Breed	# of blood samples collected total
111968	4 y	American Bulldog	9		101057	4 y	English Bulldog	1
152217	2 y	American Bulldog	5		105888	4 y	English Bulldog	1
158214	2 y	American Bulldog	5		145788	3 y	French Bulldog	5
163365	2 y	American Bulldog	4		159740	3 y	French Bulldog	2
159948	2 y	American Bulldog	2		114487	4 y	French Bulldog	1
101197	5 y	American Bulldog	1		143867	5 y	Labradoodle	4
115084	7 y	American Bulldog	1		162470	2 y	Labrador Retriever	4
159798	3 y	American Pitbull	2		97411	5 y	Mastiff	7
162482	6 y	Australian Shepherd	1		110910	3 y	Mastiff	4
151603	4 y	Belgian Malinois	3		102331	5 y	Pitbull	4
106638	6 y	Brittany	2		151589	3 y	Pitbull	3
151851	4 y	Cane Corso	3		158382	3 y	Pitbull	2
153612	3 y	Corgi	3		102284	4 y	Pitbull	2
87549	5 y	Dachshund	1		155705	4 y	Rottweiler	4
142897	5 y	English Bulldog	6		153703	3 y	Schnauzer	2
139554	4 y	English Bulldog	5		146453	3 y	Sheltie	2
146986	4y	English Bulldog	4		115181	3 y	Shetland Sheepdog	5
163455	2 y	English Bulldog	2		75064	9 y	Shetland Sheepdog	1
141600	3 y	English Bulldog	2		84277	10 y	Shetland Sheepdog	1
141941	2 y	English Bulldog	1		87458	7 y	Siberian Husky	3
147259	2 y	English Bulldog	1		163913	5 y	Siberian Husky	2
158809	2 y	English Bulldog	1		157231	4 y	Weimaraner	3

*The age of the dog at the time the first blood samples were collected.

9
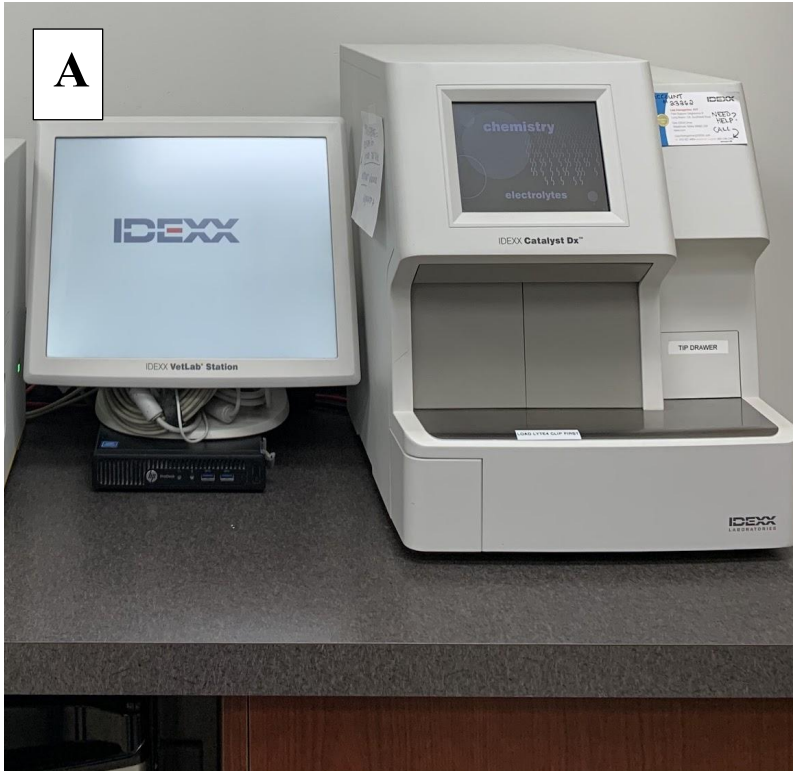
Figure 1: The CIA progesterone analyzer (A) and ELFA progesterone analyzer (B) used in this research.

Results:

The CIA had a lower and upper detection limit of 0.2 ng/ml and 20 ng/ml, respectively. Results outside of the detection limits for CIA were excluded from analysis. In addition, the ELFA had two samples with [P4] >50 ng/ml and these were excluded as outliers.

The Pearson's r values from the Passing-Bablok regressions were all high, but all three comparisons varied from the line of identity (**Figures 2-4**). The comparison furthest from the line of identity was between CLIA and ELFA while the comparison closest to the line of identity was between CIA and ELFA.

Histograms from results of each assay demonstrated that approximately half the results were above or below 5 ng/ml. Due to the variability seen in the residuals plots from the entire data set, two residuals plots were created (≤5 ng/ml and >5 ng/ml) for each assay comparison. If the results from one assay was >5 ng/ml and the results from another assay was <5 ng/ml, this data was not included on the residuals plots. For this reason, the ≤5 ng/ml residuals plots had more data points than the >5 ng/ml residuals plots. The residuals plot for the CLIA and CIA comparison and the ELFA and CIA comparison demonstrated an increase in residual spread at progesterone values >5 ng/ml (**Figures 2B-C and Figures 4B-C**). This indicates that while our model is very accurate for measuring low progesterone concentrations on these three machines, as progesterone concentrations increase >5 ng/mL, the model become less accurate. It is also worth noting that the true concentration of progesterone can only be determined by running a spiked sample with a known concentration of progesterone, and this was not done in this study.
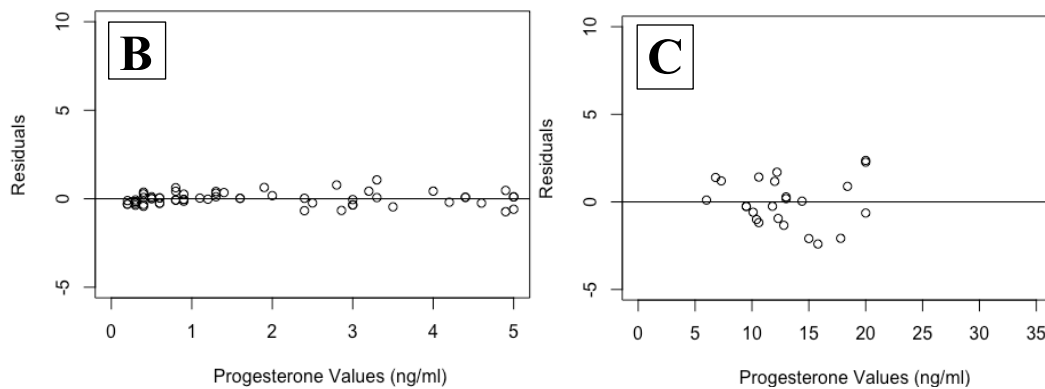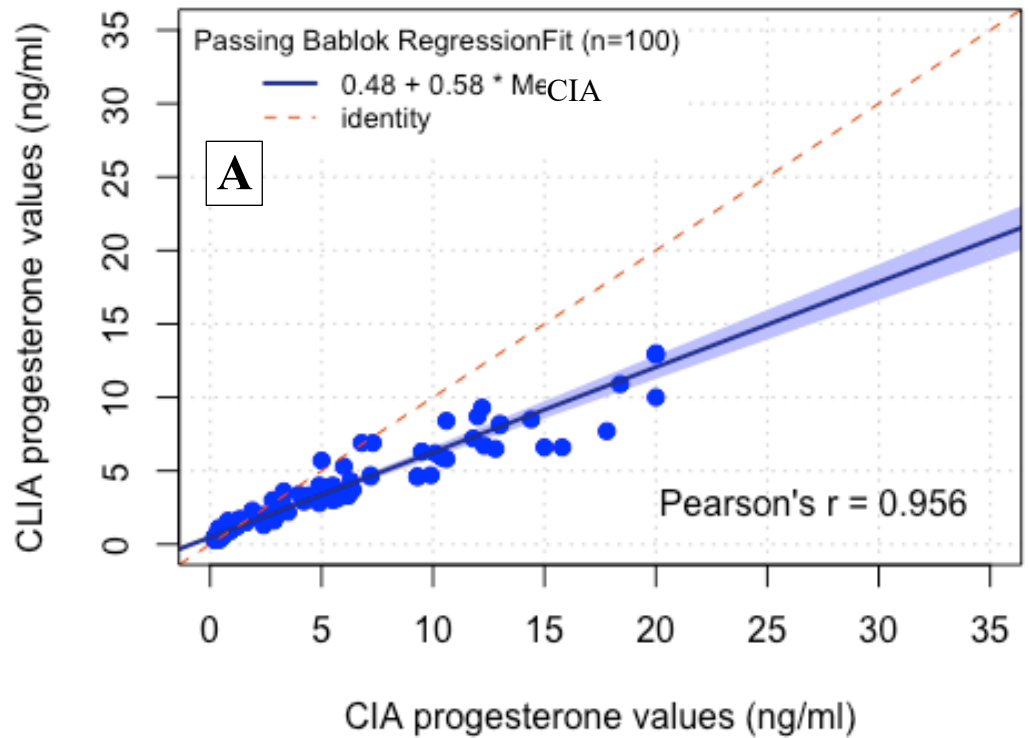
Figure 2: A: This plot illustrates the correlation found through the Passing-Bablok method between the reference lab's CLIA progesterone assay and the inhouse CIA progesterone assay (n=100). **B:** A residuals analysis between CLIA reference laboratory's progesterone assay and the inhouse CIA progesterone assay for progesterone values ≤5 ng/mL. (n=60) **C:** A residuals analysis between CLIA reference laboratory's progesterone assay and the inhouse CIA progesterone assay for progesterone values >5 ng/mL (n=24).
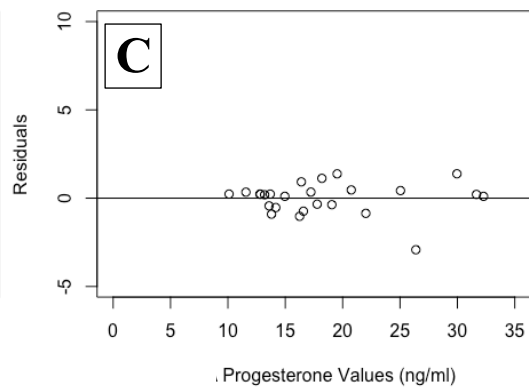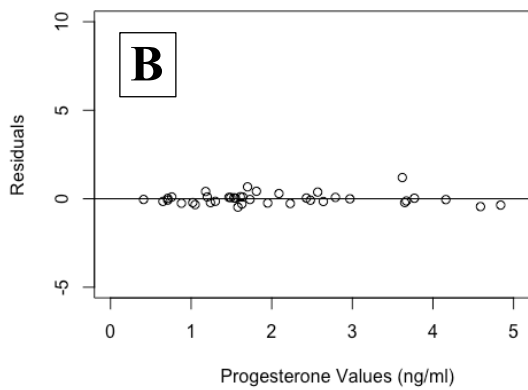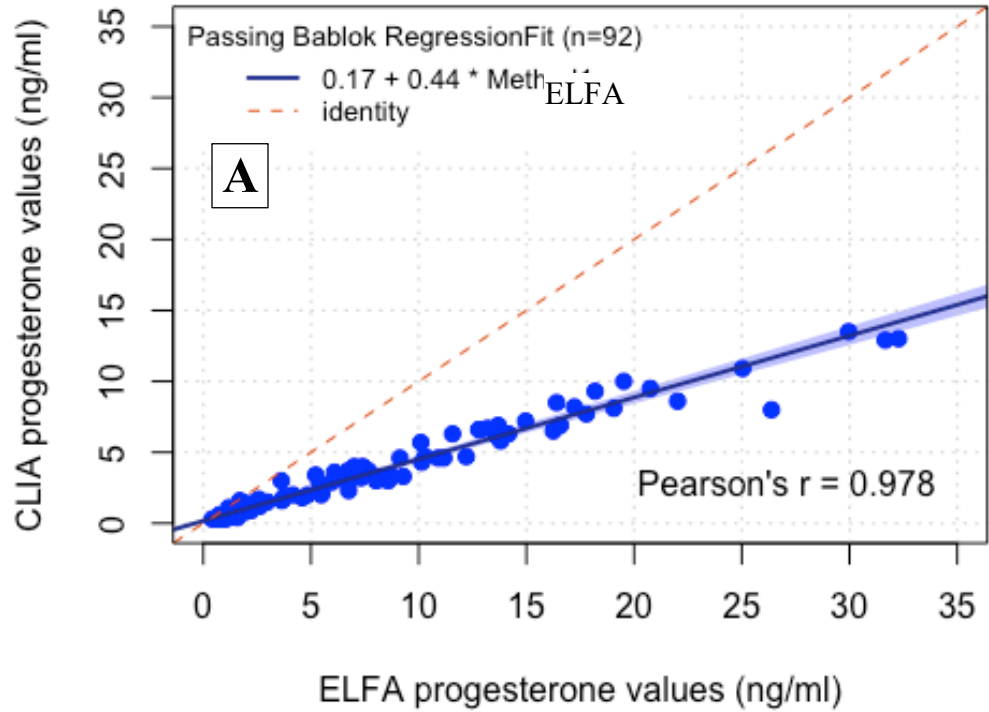
Figure 3: A: This plot illustrates the correlation found through the Passing-Bablok method between the reference lab's CLIA progesterone assay and the in-house ELFA progesterone assay (n=92). **B:** A residuals analysis between CLIA reference laboratory's progesterone assay and the in-house ELFA progesterone assay for progesterone values ≤5 ng/ml. (n=39) **C:** A residuals analysis between CLIA reference laboratory's progesterone assay and the in-house ELFA progesterone assay for progesterone values >5 ng/ml (n=27).
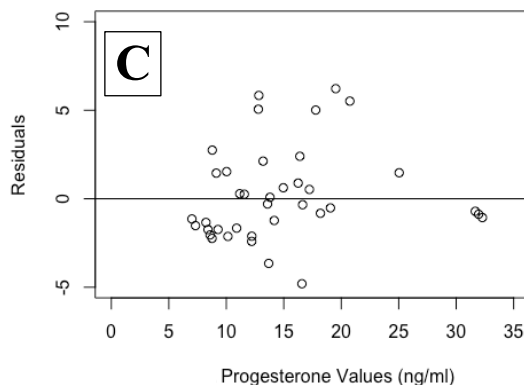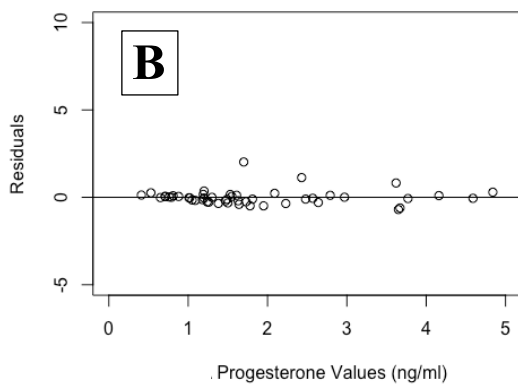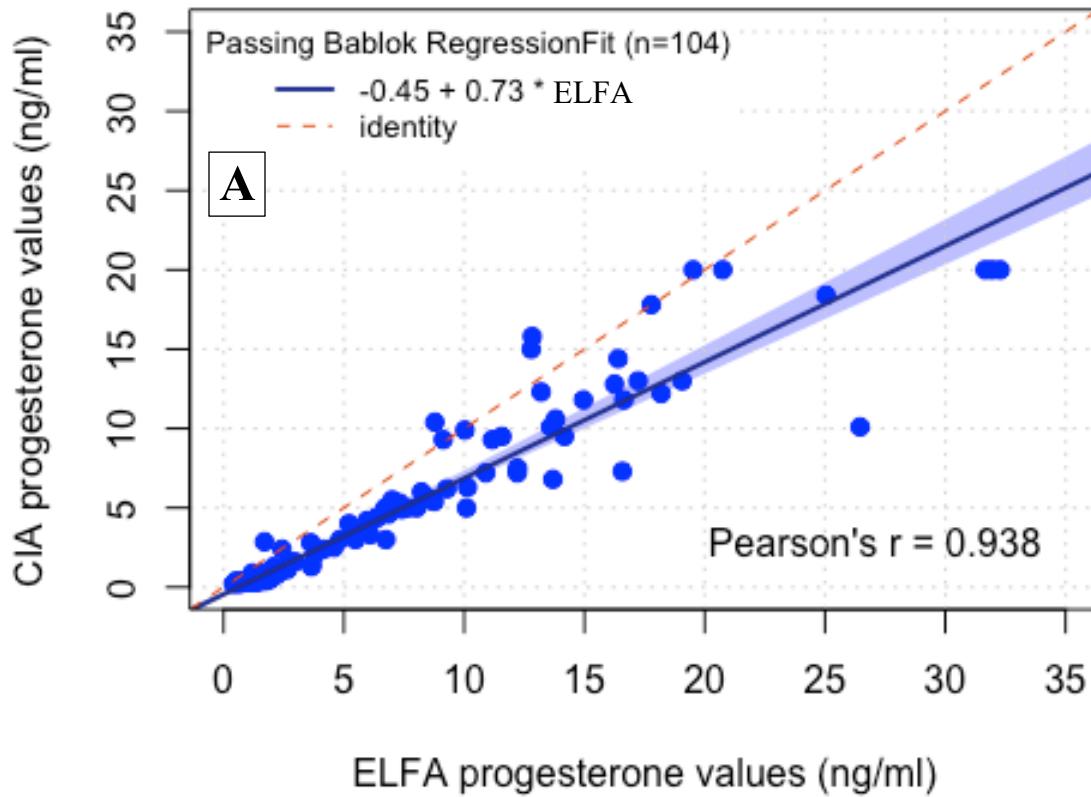
Figure 4: A: This plot illustrates the correlation found through the Passing-Bablok method between the in-house CIA progesterone assay and the in-house ELFA progesterone assay (n=104). **B:** A residuals analysis between in-house CIA progesterone assay and the in-house ELFA progesterone assay for progesterone values ≤5 ng/ml. (n=49). **C:** A residuals analysis between in-house CIA progesterone assay and the in-house ELFA progesterone assay for progesterone values >5 ng/ml (n=39).

Discussion:

Previous research has compared radioimmunoassays (RIA) to non-radioactive immunoassays [24,25,32,38]. In a pair of studies on canine progesterone, Chapwanya compared the RIA to the CLIA and Brugger compared the RIA to the ELFA and both reported a high Pearson correlation coefficients (r=0.98 and r=0.995, respectively)[32,38]. Liquid chromatography mass spectrometry (**LC-MS**) has been proposed as a gold standard alternative to RIA[30,39]. While LC-MS is not an immunoassay, it has been compared to both CLIA and CIA for [P4] and found to have a high Pearson correlation coefficient with LC-MS (r=0.99 and r=0.99, respectively)[30]. The current study did not include a comparison to RIA or LC-MS because these methods are not available in clinical practice. However, the findings of the current study evaluating CLIA, ELFA and CIA yielded a high Pearson r for all comparisons (Figures 2-4). The comparison between CLIA and ELFA was the highest correlation between the three assays evaluated in this study (r=0.978) (Figure 3). This may be because both CLIA and ELFA use detection methods that are more sensitive (luminescence and fluorescence) than colorimetric methods and can detect lower values of [P4][37]. An additional advantage of ELFA is that it can be run in-house, whereas CLIA is only available through reference laboratories[31].

While each assay demonstrated a high degree of correlation with each other, the assay results were not the same. For instance, CLIA yielded consistently lower [P4] values >5 ng/ml when compared to CIA (CLIA = 0.58*CIA+0.48) and ELFA (CLIA = 0.17*ELFA + 0.44) (Figures 2-3). This indicates that results from different assays need formulaic adjustments to be compared. However, these correlations may differ between assays due to variations in calibration or lots of reagents. As the [P4] increased, the

amount of variance in results also increased (Figures 24). Similarly, to our findings, Chapwanya's comparison of CLIA (Immulite) and RIA appeared to demonstrate an increase in variance as the mean values of [P4] increased past 2 ng/ml[32]. It is important to mention that the primary uses of [P4] in canine reproduction (e.g., timing for insemination or elective C-section) need to reliably measure low [P4] ($\leq$5 ng/ml), which was highly correlated in all three assays.

Conclusions/Future Studies:

The key findings of this study were that at low [P4], all three assays were highly correlated, but at higher concentrations, they showed more variability. Since the important uses of progesterone all occur at lower concentrations, this wasn't a major concern. Additionally, we found that the CLIA that Idexx Reference Labs use (Immulite 2000) gives consistently lower results than both the ELFA (Mini Vidas) and the CIA (Catalyst Progesterone) assays. Despite these lower readings, results from one assay can be compared to the other two assays through formulaic adjustments created by simple linear regression.

Since RIA is becoming less common, and since several studies have proposed LC-MS as an alternative gold standard, it will be useful to compare these two[29,40]. LC-MS doesn't rely on antibody binding to antigen, but instead measures substances of interest by analyzing the actual structure of the molecules themselves[21]. Therefore, it often times is more accurate than RIA[21]. Comparing some assays to LC-MS has been previously reported, such as in Schooley's comparison of CIA and CLIA to LC-MS[30].

Another potential area to research would involve comparing the results from various commercial immunoassay to LC-MS or spiked samples with known concentrations of progesterone. While this wasn't done in this study, both LC-MS and spiked samples are tools that can be used to determine the true accuracy of the various clinical immunoassays.

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