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# Evaluation of a non-radioactive rapid test for the determination of serum progesterone in the mare

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ABSTRACT

The determination of progesterone from mares' serum plays a decisive role in diagnosing estrus cycle disorders or luteal insufficiency. To date, no measurement methods are available for rapid quantitative diagnosis of serum progesterone in the mare that would allow results to be available within a two-hour time frame. The present study will evaluate a commercial enzyme-linked fluorescent assay, the mini VIDAS device (bioMérieux, Nürtingen, Germany). Serum was prepared from the blood samples of one hundred and seven mares, divided into two aliquots, and stored at –20°C. Subsequently, comparative progesterone measurements were carried out using radioimmunoassay (RIA) and mini VIDAS, with RIA as the reference standard. The results show a strong positive correlation between the two measurement methods (Spearman rank correlation $rs = 0.96381$ ($p < 0.05$). The tests confirm that the mini VIDAS can be used for equine serum progesterone concentration determination and provides reliable results in less than an hour. For accurate estrus cycle diagnostics, high precision in the low measurement range is essential, as even minor deviations can result in ovulation misdiagnosis. Despite the high correlation of the measuring methods, deviations from the reference method can be observed in the low measuring range of the mini VIDAS, which suggests potential limitations of this study.

## 1. Introduction

Progesterone concentration determinations are an essential part of the diagnosis of estrus cycle disorders in the mare [1,2]. Progesterone originates from the luteal cells of the corpora lutea. Measuring serum progesterone concentration is used as a diagnostic indicator of ovulation and physiological luteal function in the mare. Values of >1 ng/ml suggest active luteal secretion of progesterone [3,4]. The average values for the follicular phase are 0.02-0.04 ng/ml [5], other studies show values permanently below 1 ng/ml during estrus [3,6]. However, there is a high individual variation in serum progesterone concentrations in mares, even in the absence of endocrine disorders, which must be considered when interpreting the results [7].

In the first 40 days of pregnancy, the primary corpus luteum is the only source of progesterone [8–10]. In this phase, the progesterone concentration increases after ovulation, peaks between day five to ten [6,11], and then decreases moderately until the formation of secondary corpora lutea, when a second increase at day 38-40 is observed [12,13]. The progesterone concentration varies between 4-10 ng/ml in the first 90 days of pregnancy [11,14]. Recent studies recommend monitoring serum progesterone levels five days after ovulation to detect mares with impending embryonic death due to luteal insufficiency [15,16]. To this date, no definitive required minimum progesterone concentration could be determined [17], though serum progesterone concentrations >4 ng/ml have been suggested to be sufficient to maintain pregnancy prior to luteo-placental shift in mid-gestation [18,19]. Multiple measurements might be necessary [7,18]. In these cases, progestin supplementation may be indicated [20,21].

To date, there are no rapid quantitative measurement methods providing results within two hours available for routine laboratory diagnostics in the horse as there are in the bitch, which makes early diagnosis of fertility disorders difficult in practice [5]. The methods used to measure the progesterone concentration are usually laboratory-based enzyme immunoassay (EIA, ELISA) or radioimmunoassays (RIA). However, using radioactive material and the duration (approx. 24 h) of this method may be seen as a disadvantage [22,23].

In recent years, the mini VIDAS multiparametric immune analyzer (bioMérieux, Nürtingen, Germany), developed initially for use in

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humans, has been introduced for small animal practice for quantitative progesterone determination using the enzyme-linked fluorescent immunoassay (ELFA) technique [24,25]. Within one hour, progesterone concentration can be determined in a laboratory. Once a measurement method has been validated for humans or a certain animal species, it cannot be transferred to another species without validation. Even if the structure of progesterone is species-independent, erroneous measurement can occur due to matrix effects [26]. For this reason, this study aims to evaluate the automated rapid non-radioactive determination of progesterone from mare serum using the mini VIDAS.

## 2. Material and methods

### 2.1. Collection and processing of blood samples

Blood samples from 107 mares were analyzed. Retained samples from the Veterinary Clinic for Reproductive Medicine and Neonatology of the Justus Liebig University Giessen and blood taken from mares for diagnostic purposes were used. Consent for their horses' data to be used in the study was provided by all owners. Venipuncture was performed in adult horses of different reproductive stages (Table 1). Blood was taken from the jugular vein using an S-Monovette® (Sarstedt AG & Co., Nümbrecht, Germany) with coagulation activator for the subsequent preparation of serum. Samples from the period from May 2021 to February 2022 were used. The use of the blood was approved by the responsible regional council in Giessen (KTV 7-2017).

### 2.2. Determination of serum progesterone concentration

After collection, the blood samples were centrifuged for five minutes at 4000 rpm and at room temperature. The serum of each animal was then divided into two aliquots and stored at -20°C until progesterone determination (tube, 5 ml: 75 × 12 mm, PP, Sarstedt AG & Co., Nümbrecht, Germany).

As a reference method, progesterone was determined in one of the two aliquots using the radioimmunoassay (RIA) of the endocrine laboratory of the Veterinary Clinic for Reproductive Medicine and Neonatology of the Justus Liebig University Giessen, following the procedure described by Hoffmann et al. 1973 [27]. The application of this method to equine blood samples has been previously published [28]. The antiserum applied was obtained after immunization against 11α-hydroxyprogesterone hemisuccinate-BSA and exhibited the following cross-reactivity: Progesterone: 100 %, pregnenolone: 0.69 %, 17α-OH-progesterone: 0.49 %, testosterone: 0.37 %, androstenedione, estradiol-17b, estrone, and cortisol: <0.01 % [24]. Prior to RIA, the serum was twice extracted with hexane. The pooled extracts were dried and redissolved in phosphate buffer with 0.1 % bovine serum albumin. Antibody-bound and free steroids were separated using an activated carbon suspension. The determination was carried out using a tritium-labeled tracer. The intra- and interassay coefficients of variation are 8.8 % and 8.9 % respectively [29].

The mini VIDAS measured the second aliquot based on the ELFA test principle (Enzyme-Linked Fluorescent Assay) [22], which combines a competitive immunoassay with subsequent fluorescence detection [18]. The measuring range is between 0.25 and 80 ng/ml [17]. To run up to twelve samples takes approximately 45 minutes. Sample preparation and measurement were carried out according to the manufacturer's instructions.

**Table 1**
Reproductive status of the mares whose blood was used for test evaluation.

| | Total | Pregnant | Not pregnant (estrus and diestrus) | Unknown pregnancy status |
|---|---|---|---|---|
| Number of mares | 107 | 32 | 74 | 1 |

### 2.3. Data analysis

The Spearman rank correlation and the Passing-Bablok regression were used to compare the methods. A CUSUM test for linearity was also carried out for the Passing-Bablok regression. The calculation was done using the software SAS 9.4, 2013 (SAS Institute Inc., Cary, NC, 27513-2414, USA), and the significance level was set to $\alpha = 0.05$.

## 3. Results

There was a strong positive correlation between the two measurement methods (Spearman rank correlation $rs = 0.96381$ ($p < 0.05$). The equation of the Passing-Bablok regression was RIA = 0.03807 + 0.49286 * VIDAS, from which a proportionality factor of 0.49 can be derived. This factor can be used to adjust the VIDAS value to the reference range of the RIA without determining a method-specific reference range.

The 95 % confidence interval for the slope did not include 1, i.e., a proportional difference between the two methods can be assumed here.

The 95 % confidence interval for the intercept included 0, indicating no systematic difference between the two methods. The CUSUM test for linearity was not significant, with $p = 0.3021$. Therefore, it can be assumed that the two measurement methods are linearly related (Fig. 1).

If only the values of the pregnant animals were used for the analysis, the Spearman rank correlation was 0.75069 ($p < 0.05$). The agreement between the measurement methods was significantly higher for non-pregnant animals, with a correlation coefficient of 0.96304 ($p < 0.05$).

## 4. Discussion

The rapid measurement of progesterone in mare's serum can facilitate monitoring of the estrus cycle and can help to improve diagnostics in cases of cycle irregularities or luteal insufficiency. When interpreting the values, the case history and the clinical findings must be considered [30]. If transrectal reproductive tract palpation is not possible due to the size of the equine, determination of progesterone in blood samples can be carried out instead for the investigation of ovarian status.

It was shown that the mini VIDAS is very well suited for determining serum progesterone in horses. The results from the evaluation of mini VIDAS for the determination of progesterone in canine serum samples already demonstrated how important species-specific testing is. These showed that results from mini VIDAS exhibit a strong positive correlation with the RIA. However, the mini VIDAS significantly overestimated progesterone concentrations compared to the reference method. Instead of determining method-specific reference values, a proportionality factor was introduced with which the results of the mini VIDAS can be adjusted. The proportionality factor for samples from dogs is 0.8. It should be checked regularly by comparative measurements, as the manufacturers of such devices may occasionally make changes to the method, but these are primarily geared towards use in humans [24,31]. In the study presented here, a proportionality factor of 0.49 was calculated for the horse. Measurement results from the mini VIDAS device can be multiplied by this factor so that the usual reference range of the RIA can be used.

When looking at the 95 % confidence interval, it can be seen that the consistency between the two methods decreases with higher values. There are two main possible causes for this. One possibility is that one or both methods have a problem with linearity. The measuring range of the RIA is set to concentrations between 0.1 and 8 ng, in which the linearity of the method was confirmed. For measured values in the upper measuring range or above, the measurements were repeated with a reduced sample volume so that a severe linearity problem can be ruled out for the RIA.

Another explanation could be a different cross-reactivity between the antisera used in mini VIDAS and the RIA. During the cyclic luteal

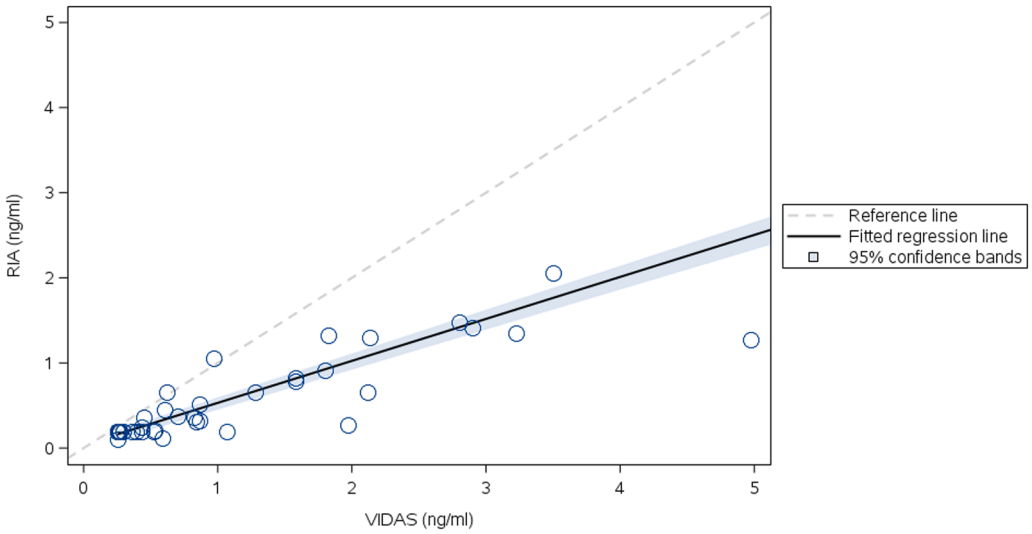


**Fig. 1.** Comparison of the two progesterone measurement methods in ng/ml from mare's serum. Passing-Bablok regression line of all values with 95 % confidence interval. The reference line shows the course of the straight line if both measurement methods could be used without a proportionality factor and in the same reference range.

phase and in early pregnancy, progesterone of luteal origin dominates by far as the target molecule of these assays in the systemic blood of the mare. With the massive onset of placental steroid synthesis around day 70-80, C(21)-steroids structurally related to progesterone increasingly circulate in the maternal blood alongside estrogens and reach extraordinarily high concentrations in the further course of pregnancy. With the disappearance of the corpora lutea, pregnancy in the mare is increasingly maintained by progestagens of placental origin, whereby progesterone is hardly measurable with specific mass spectrometric methods from mid-pregnancy onwards. Thus, the "progesterone pregnancy profiles" established using immunological methods therefore increasingly result from the cross-reactivity of the antisera used with the placental C (21) steroids as luteal progesterone production decreases, and immunological methods can thus differ considerably with regard to the progesterone concentrations measured in advanced stages of pregnancy [32]. It is, therefore, to be expected that the problem of poorer consistency between the two compared methods will increase from the second trimester of pregnancy if it is due to different cross-reactivity of the antisera. Further studies comparing early and late pregnancy could verify this hypothesis.

For practical use in estrus cycle diagnostics, high accuracy is required in the low measuring range of the method. Even a slight deviation in the result can lead to misdiagnosis regarding the time of ovulation. Looking at Fig. 1a, deviations from the RIA value can be seen in the low measuring range. If this is an inaccuracy in the measurement method, this represents a limitation for practical use. Still, the mini VIDAS shows the highest correlation with the reference standard in the low measuring range.

Elevated progestogen levels in the blood of mares in late pregnancy could indicate placental pathology, so the device could also be used in this area [33,34]. Due to the cross-reactivity possibly deviating from the RIA, further investigations would be necessary to determine cut-off values.

## 5. Conclusion

The mini VIDAS is a suitable method for serum progesterone determination in mares. Using the proportionality factor established in this study, it provides reliable results in non-pregnant mares that are consistent with the reference method in a short time. Despite the high

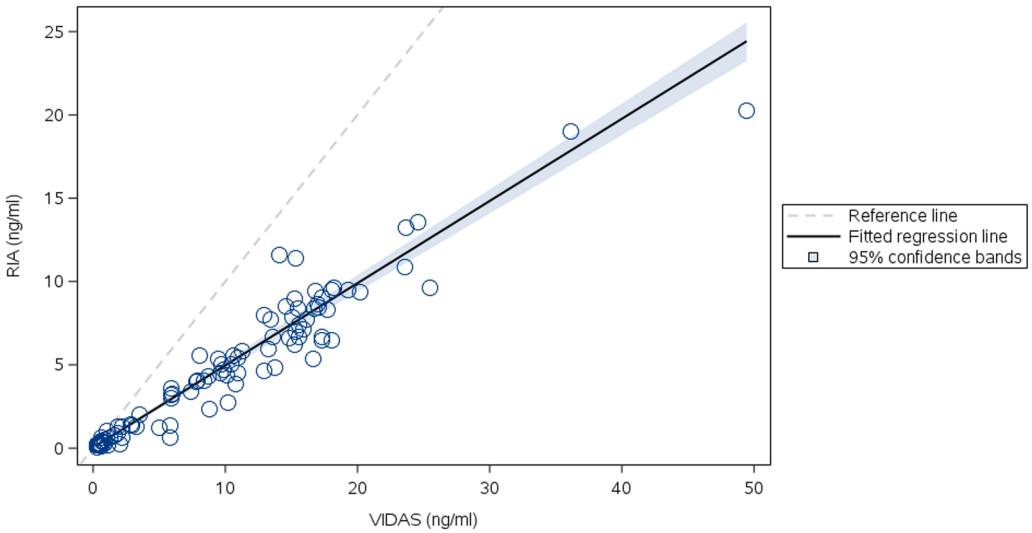


**Fig. 1a.** Detail of Fig. 1, showing the axis section below 5 ng/ml in detail.

correlation between the measurement method and the reference method, the inaccuracy in the low measurement range represents a potential limitation for clinical use.

## CRediT authorship contribution statement

**L Längerer:** Writing – review & editing, Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **G Schuler:** Writing – review & editing, Validation, Resources, Methodology, Formal analysis, Conceptualization. **K Büttner:** Writing – review & editing, Visualization, Validation, Formal analysis. **A Wehrend:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

## Declaration of competing interest

None of the authors has financial or personal relationships that could inappropriately influence or affect the content of the work.

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