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Serum progesterone profile in the oestrous cycle of mares determined using immuno chemiluminescence (CLIA)

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Summary: Six cross-bred mares (Mangalarga, Quarter Horse, Apaloosa, Criollo) were used to (1) quantify the serum progesterone (P4) levels during the oestrous cycle of mares using chemiluminescence (CLIA), (2) compare the P4 profile quantified using CLIA and the follicular dynamics data obtained using ovarian ultrasound (US), and (3) validate the CLIA methodology with an enzyme-linked immunosorbent assay (ELISA). The horses were between 10 and 18 years old, body weight ranging from 350 to 490 kg, and with a body condition score of 3.5. We conducted the study starting from the day of ovulation until the next ovulation was detected using US examinations. Blood samples were collected at intervals of two days and stored at -20 °C for the determination of P4 using ELISA and CLIA. Concomitantly, the ovaries were scanned daily using US to monitor the follicular dynamics. The length of the oestrous cycle (interval ovulation to ovulation) varied between 22 and 27 days. The results of the P4 concentration were analysed using Pearson's χ^2 and Kolmogorov-Smirnov tests. The P4 concentration in the follicular phase ranged from 0.38 to 1.91 ng/mL (CLIA) and 0.81 to 3.26 ng/mL (ELISA), and the concentrations in the luteal phase were 8.69 to 29.05 ng/mL and 11.84 to 25.16 ng/mL, respectively. The P4 concentration $\geq$ 5.48 ng/mL was indicative of the luteal phase (days 3 to 15 of the cycle) and $<$ 5.47 marked the follicular phase of the oestrous cycle (day 16 until the next ovulation). The dimensions (diameter) of the largest follicle and corpus luteum varied between 35.0 and 47.5 mm and 14.2 and 43.7 mm, respectively. A follicular wave was observed during the oestrous cycle of the animals. The follicular and luteal phases of the oestrous cycle were established based on P4 concentrations after the analysis using the χ^2 and Kolmogorov-Smirnov adherence tests. The Pearson's χ^2 test results indicated a p-value $<$ 0.05, presenting different frequencies and numerical values between ELISA and CLIA; however, the curves of both the analyses depicted the same trend. Therefore, after adjustment, the Kolmogorov-Smirnov adherence test (KS at 95 % probability) was performed where the maximum difference between the observed and estimated frequencies was compared and divided by the number of observations. The values proved to be adherent, with the same tendency in the resulting curve. The CLIA proved to be an efficient method for determining the serum P4 in mares, as it determined the P4 profile, showing adherence to the ovarian findings verified using US examinations. Therefore, CLIA can be used as an alternative method because it has been proved to be an efficient, low-cost and rapid method.

Keywords: chemiluminescence, enzyme-linked immunosorbent assay, oestrous cycle, immuno chemiluminescence, mares, progesterone

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Introduction

The biotechnological techniques related to animal reproduction (e.g. timed artificial insemination, timed embryo transfer, superovulation, oestrous cycle and ovulation synchronisation) have made significant progress in the last decade in improving the reproductive efficiency. In this context, the methodologies for the determination of reproductive hormones have also contributed to the advancements in this research field.

Hormonal tests for the determination of progesterone (P4) profiles showed a marked development at the end of 1970s (*Hoffmann* and *Oettel* 1976, *Kohen* et al., 1979), with radioimmunoassay (RIA) being one of the best-known methods till now. The RIA for the determination of the P4 serum concentration is based on the use of radioactive isotopes (*Parker* 1981), particularly in dairy cows (*Kozicki* et al. 1984). However, RIA is gradually being excluded because of the use of radioactivity. At the same time, enzymatic assays were developed, with an emphasis on the enzyme-linked immunosorbent assay (ELISA) (*Engvall* and *Perlmann* 1971) and enzyme immunoassay (*Felgner* 1982).

At the end of 1970s, a rapid, low cost method, applicable on a large scale, known as a chemiluminescent immunological assay (immuno chemiluminescence: CLIA) was developed (*Kohen* et al. 1979, 1981). The CLIA is based on the emission of light (without heat emission) released from a chemical reaction that generates light energy. Interestingly, the CLIA methodology has not followed similar trends in veterinary medicine

because it has not been often used to date. *Volkmann* (2006) and *Tahir* et al. (2013) determined P4 concentrations (in canines) using the CLIA methodology and concluded that it is an efficient method to be applied in veterinary medicine. *Xiao* and *Da* Zhao (2010) used CLIA to determine the profile of P4 in the oestrous cycle and during pregnancy in Meriones unguiculatus. *Fantini Filho* et al. (2004) determined the P4 concentration using CLIA in beef cows (during pregnancy) on days 7, 13 and 24 after artificial insemination. The CLIA was proved to be a useful methodology capable of determining the P4 profile of dairy cows during the oestrous cycle. *Kozicki* et al. (2018) demonstrated that a serum P4 value < 5.48 ng/mL is indicative of the follicular phase and ≥ 5.48 ng/mL of the luteal phase in cows, which was further confirmed by following the oestrous cycle upon daily examination of ovarian ultrasound (US).

Therefore, we aimed in the present study to (1) determine the concentration of P4 quantitatively during the oestrous cycle of mares using the CLIA methodology, (2) compare the P4 profile determined using CLIA and correlate it with the follicular dynamics data obtained from ovarian examination (US), and (3) further validate the methodology using ELISA.

The study was conducted at an experimental farm located at the following coordinates: latitude, 25° 39′ 31″ S; longitude, 49° 18′ 32″ W. Six cross-bred mares (Mangalarga, Quarter Horse, Apaloosa, Criollo) which were multiparous and non-lactating, between 10 and 18 years old, with a body weight ranging from 350 to 490 kg and a body condition score of 3.5 were used. Mares were fed with a specific horse feed (2 kg of concentrate/animal/day: ProEquine Agraria® [12% protein, 12% fibre, 14% mineral matter and 2.5% ether extract], Guarapuava, Paraná, Brazil), alfalfa hay and pasture (Cynodon tifton), along with water and mineral salt ad libitum.

Mares were previously examined for ovarian cyclicity (presence of follicles larger than 35 mm or corpus luteum) and were free of clinical endometritis. No hormones were administered to synchronise or induce ovulation in order to initiate the study. Instead, the animals were expected to reach oestrous naturally.

Ovarian data collection

Data was collected through US examination of the ovaries to monitor the follicular dynamics during the oestrous cycle. Day zero (d0) of the cycle was considered to be when the ovulation of the dominant follicle was detected. A complete oestrous cycle was marked when the animals ovulated again after d0. The ovaries were scanned daily using an US device (SonoScape A5, linear probe, Shenzhen, China) and the ovarian findings were assigned to the ovarian maps, following the changes in the gonads during the interval between the two consecutive ovulations.

Blood harvest and serum handling

Blood was collected (puncture of the vena jugularis) using vacuum tubes and needles (Vacutainer® type) after scanning the ovaries. Blood samples were collected at intervals of two days in two different flasks for subsequent analysis using CLIA and ELISA. After collecting the blood samples, the tubes were placed in a water bath at 37 °C until centrifugation (5000 rpm) to separate the serum. After centrifugation, the serum was placed in sterile Eppendorf tubes, labelled and stored at -20 °C until P4 determination.

Progesterone determination

Using CLIA

Serum samples for P4 determination were analysed in a commercial laboratory (Curitiba, Paraná, Brazil) using the CLIA methodology. Accordingly, the CLIA commercial kit (Architect Progesterone, Wiesbaden, Germany) was used. The sensitivity for P4 ranged between 0.10 and 40.0 ng/mL. The Architect Progesterone test was applied to measure P4 concentrations with an analytical sensitivity of ≤ 0.1 ng/mL.

Using ELISA

The serum P4 concentration values were determined through ELISA using the Progesterone ELISA kit (DRG Progesterone CLA 4663, Marburg, Germany) in a laboratory of the Pontificia Universidade Catolica of Paraná. Therefore, a spectrophotometer (Epoch 2, Biotek, Winooski, Vermont, USA) was used at a wavelength of 450 nm for device calibration.

Statistical analysis

Data analysis was performed using the IBM SPSS Statistics version 25 (USA). A pilot test was initially performed to check the absorbance of CLIA and ELISA, and the results were compared to those of the RIA. The P4 concentrations on each day of the oestrous cycle of all animals (determined using CLIA and ELISA) were compared using the χ^2 adherence test (Pearson's χ^2) ($P < 0.05$, Equation: Eq. 1). This test compares two distributions, analysing the possible divergences between the observed and expected frequencies.

$$x^2 = \sum_{i=1}^{n} \frac{(o_i - e_i)^2}{e_i} \quad \text{(Eq. 1)}$$

Where *oi* is the P4 concentration on each day of the oestrous cycle of animals obtained using CLIA, ELISA; and *ei*, using RIA.

The values were standardised by adjusting the ratio between the sum of ELISA concentrations and that of CLIA concentrations. The CLIA correction factor (FCC), which is the ratio between the two tests (Eq. 2), was obtained with this operation, resulting in an FCC = 2.536129. When the FCC had been established, the original mean values obtained through CLIA were multiplied by the FCC to compare the test curve to the standard (ELISA), which is an adaptation of the method for the hormonal dosage of the species.

$$FCC = \frac{\sum ELISA}{\sum CLIA} \quad \text{(Eq. 2)}$$

After adjusting the original CLIA values (upon FCC multiplication), they were compared with the standard ELISA values using the Kolmogorov-Smirnov adherence test ($p = 0.05$; Eq. 3). The latter compared the maximum relative difference between the observed and estimated accumulated frequencies.

$$D_n = \sup_x |F(x) - F_n(x)| \quad \text{(Eq. 3)}$$

This function corresponds to the maximum vertical distance between the graphs of F(x) and Fn(x) over the amplitude of the possible x values. The maximum *Dn* is compared with the critical value obtained using Eq. 4, which is considered adherent if the value calculated is less than the critical value (*Dcritical*). As the maximum *Dn* was lower than *Dcritical*, we infer that the distributions did not differ significantly, thereby presenting similar distributions (Eq. 4).

$$Dcritical = \frac{1{,}36}{\sqrt{n}} \quad \text{Eq. 4}$$

Results

Ovarian examinations were performed during the oestrous cycle using an US device to monitor the follicular dynamics, and the dimensions of the largest follicle and corpus luteum were recorded. The interval between the two consecutive ovulations ranged between 22 and 27 days (Fig. 1).

The mean absorbance value obtained using ELISA was 3.9837 times greater than that obtained using RIA, while the mean value obtained using CLIA was 1.7433 times greater than that of RIA, considering that the R^2 values were high (> 0.95) and that the residuals were close to zero.

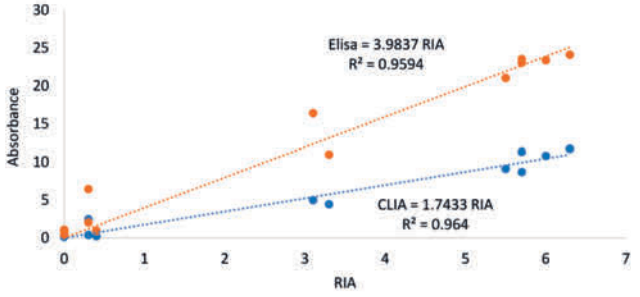

Fig. 1 Relationship of the absorbance values of ELISA and CLIA with those obtained using RIA. | *Verhältnis der Absorptionswerte von ELISA und CLIA zu den mittels RIA erhaltenen Werten.*

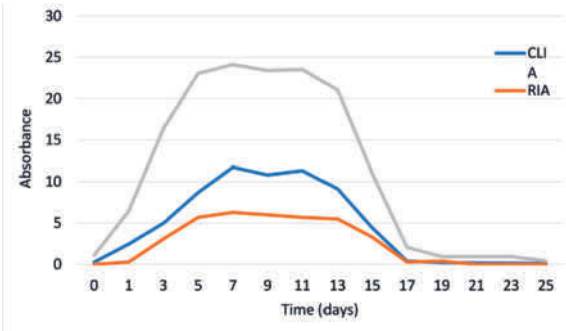

Fig. 2 Absorbance values obtained over time using RIA (*Hoffmann* et al.1973), CLIA and ELISA tests. | *Absorptionswerte, die im Laufe der Zeit mit Hilfe von RIA (Hoffmann et al. 1973), CLIA und ELISA-Tests ermittelt wurden.*

Figure 2 confirms the linear relationship between RIA and ELISA, as well as between RIA and CLIA, clearly depicting that the absorbance trends for the three tests were similar. Therefore, we infer that the pattern of behavior of the absorbance is proportional between the three methods.

Comparison made using the χ^2 test ($p = 0.05$)

The results presented in Table 1 are further depicted in Figure 3. It can be seen that the trend presented is similar but with a few outliers. This result was further confirmed using the χ^2 test ($p < 0.05$), which demonstrated a significant difference between the values obtained using CLIA and ELISA.

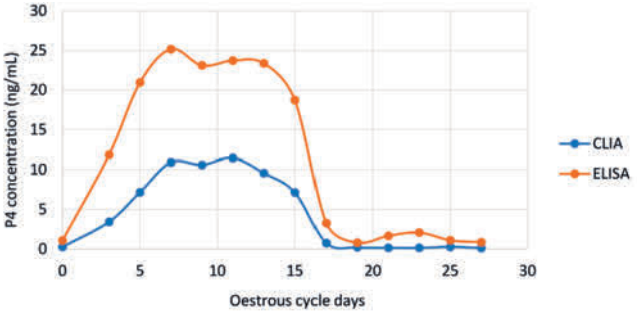

Fig. 3 Original progesterone (P4) concentration profiles observed determination using ELISA and CLIA methodologies (without the correction factor) during the oestrous cycle in cross-bred mares. | *Original-Progesteron (P4)-Konzentrationsprofile, die mittels ELISA- und CLIA-Methode (ohne Korrekturfaktor) während des Östruszyklus bei Stuten ermittelt wurden.*

Table 1 Concentration of serum progesterone (P4) determined using CLIA and ELISA in mares during the oestrous cycle in the breeding season. | *Serum-Progesteron-Konzentration (P4) bestimmt mittels CLIA und ELISA bei Stuten während des Östruszyklus in der Zuchtsaison.*

(Cycle's day)	Serum P4 concentration ng/mL – CLIA	Serum P4 concentration ng/mL – ELISA
0	0.27	1.09
3	3.43	11.84
5	7.15	20.94
7	10.9	25.16
9	10.6	23.11
11	11.46	23.72
13	9.56	23.37
15	7.12	18.73
17	0.75	3.26
19	0.22	0.81
21	0.16	1.67
23	0.15	2.08
25	0.28	1.11
27	0.14	0.86

We observed a significant difference when comparing the values obtained using ELISA and CLIA. It was necessary to generate a correction factor FCC or approximation of the curves to adjust the profile generated through CLIA, since both curves presented the same trend in their profile. Therefore, the values obtained for the direct concentration (Table 1) using CLIA were multiplied by the FCC, thereby constituting the ratio between the concentrations obtained from ELISA and CLIA tests. This was performed because of the statistical design proposed. The values were very close after the FCC application, as validated by the Kolmogorov-Smirnov adherence test. The FCC value was 2.536129, and the result applied to CLIA (Table 2) proved the similarity between the two methods (Fig. 4).

However, by the behavior of the curve, the values were corrected using FCC to approximate the curves. After the FCC was applied, Figure 4 was constructed (using the corrected values), and the Kolmogorov-Smirnov adherence test was then performed. The test showed that the curves were adherent, and the values were similar, being sufficient to apply the correction factor. Therefore, we infer that CLIA is a method that generates results similar to those of ELISA.

The statistical equality between ELISA and CLIA methods of dosing is explained in Figure 4, since there was adherence between the two curves. Therefore, it appears that the CLIA methodology reached the validation point using the gold standard method (ELISA). Therefore, it became possible to fix the P4 concentration to ≥ 5.48 ng/mL, indicating the luteal phase (from day 3 to 15 of the cycle), and < 5.47 as the follicular phase of the oestrous cycle (from day 16 until the next ovulation) (Table 2).

Figure 5 depicts the profile of the findings of monitoring follicular dynamics performed using transrectal US examinations of all mares, and Figure 6 depicts the average profile of monitoring the animals' ovaries.

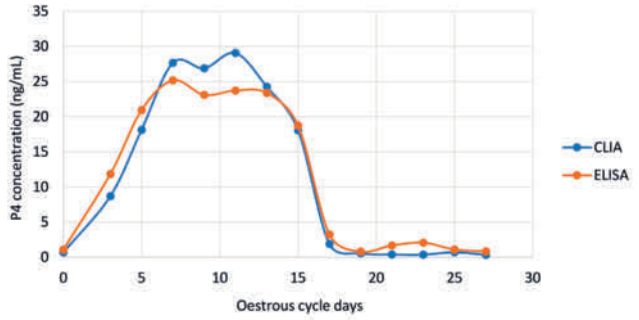

Fig. 4 The P4 concentration profiles obtained using ELISA and CLIA methodologies after applying the correction factor (CC= 2.536129) on CLIA values during the oestrous cycle in cross-bred mares. | *Die mit ELISA- und CLIA-Methoden ermittelten P4-Konzentrationsprofile nach Anwendung des Korrekturfaktors (CCF = 2,536129) auf die CLIA-Werte während des Östruszyklus bei Stuten.*

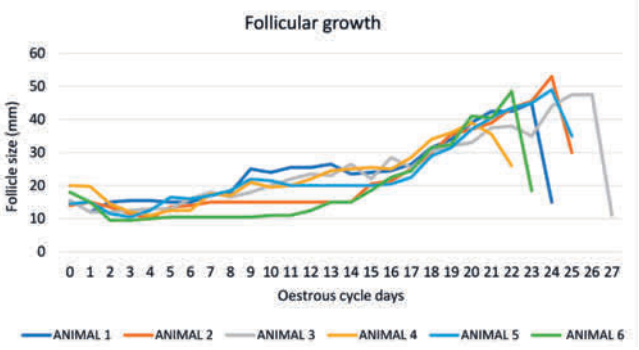

Fig. 5 Ovarian follicular dynamics in the oestrous cycle of cross-bred mares (n = 6) between day zero (= day of ovulation) until the next ovulation monitored using transrectal US examinations. | *Ovarielle Follikeldynamik im Östruszyklus von Stuten (n = 6) zwischen Tag Null (= Tag des Eisprungs) und dem nächsten Eisprung mittels transrektaler US-Untersuchungen.*

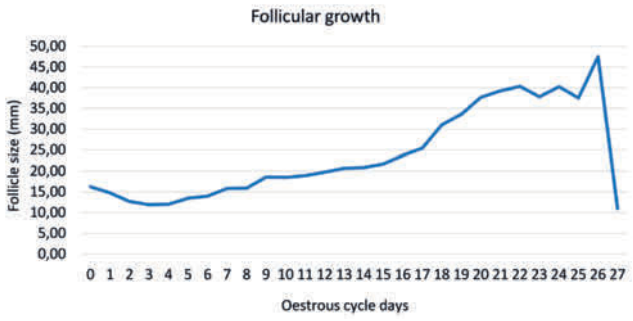

Fig. 6 Profile (average) of ovarian follicular dynamics in the oestrous cycle of six cross-bred mares between the two consecutive ovulations monitored using transrectal US examinations. | *Profil (Durchschnitt) der ovariellen Follikeldynamik im Östruszyklus von sechs Stuten zwischen den beiden aufeinander folgenden Ovulationen, die mittels transrektaler US-Untersuchungen überwacht wurden.*

Table 2 The P4 concentration after applying the Kolmogorow-Smirnow adherence test and CLIA correction factor (FCC = 2.536129) on the days of the oestrous cycle of mares. Comparison made by the Kolmogorov-Smirnov test (p = 0.05). | *Die P4-Konzentration nach Anwendung des Kolmogorow-Smirnow-Haftungstests und des CLIA-Korrekturfaktors (FCC = 2,536129) an den Tagen des Östruszyklus der Stuten. Vergleich mit dem Kolmogorov-Smirnov-Test (p = 0,05).*

Kolmogorow-Smirnow (Cycle's day)	Serum P4 concentration – ng/mL – CLIA	Serum P4 concentration – ng/mL – ELISA
0	0.69	1.09
3	8.69	11.84
5	18.14	20.94
7	27.65	25.16
9	26.89	23.11
11	29.05	23.72
13	24.25	23.37
15	18.06	18.73
17	1.91	3.26
19	0.57	0.81
21	0.41	1.67
23	0.38	2.08
25	0.71	1.11
27	0.36	0.86

Comparisan made by the Kolmogoroy-Smirnov test (p = 0.05)

Discussion

Hormonal determination has become important due to the advancements in biotechnological techniques related to animal reproduction, allowing a better understanding of reproductive physiology. Therefore, the methods for hormonal determination of P4 using RIA (*Parker* 1981), enzyme immunoassay (*Felgner* 1982) and ELISA (*Engvall* and *Perlmann* 1971) have been developed. Alternative methods have been developed limiting the application of RIA. More recently, CLIA has become widely used owing to its acceptance and applicability in human medicine. It is gradually gaining attention among researchers from other research areas, especially veterinary medicine, regarding canines (*Tahir* et al. 2013) and cows (*Kozicki* et al. 2018).

Although the ELISA methodology is a standard methodology, the use of CLIA was sought as an alternative for serum P4 determination because of its rapid execution, large-scale employability, low cost and reliability in human medicine.

Limited reports related to serum P4 determination in large animals using the CLIA methodology have been found. *Kozicki* et al. (2018) determined P4 levels during the oestrous cycle in dairy cows. Few studies have been conducted to quantify P4 in mares using the CLIA methodology. *Volkmann* (2006) and *Tahir* et al. (2013) determined P4 using CLIA in canines and concluded that the method proved to be efficient. *Xiao* and *Da* Zhao (2010) used CLIA to determine the P4 profile in Meriones unguiculatus in the oestrous cycle and during pregnancy. A more recent study was conducted in dairy cows, indicating that CLIA is a methodology capable of determining the profile of serum P4 in cows. In this research, the values < 5.48 ng/mL of serum P4 were established to define the follicular phase, and ≥ 5.48 ng/mL for the luteal phase of the oestrous cycle (*Kozicki* et al. 2018).

We decided to evaluate the absorbances obtained using RIA, ELISA and CLIA to validate the application of CLIA against ELISA. Although the values were different between the methodologies, the correlation coefficient was high, justifying the equations as the predictors of the best adjustment to the sample (Fig. 1 and 2).

Adherence was observed between the two curves, despite the differences in P4 levels, to compare the results of P4 concentrations obtained using the two methodologies (CLIA versus ELISA) (Fig. 3 and 4). The values of P4 profiles according to CLIA were initially lower than those detected using ELISA (Fig. 3, Tab. 1). Therefore, it was required to apply a constant factor, the FCC (FCC = 2.536129), in order to obtain values similar to those of ELISA (currently the standard method). After the FCC had been applied, the values allowed a good approximation of the profiles to each other (Fig. 4, Tab. 2). Therefore, it was observed that the P4 profile of the animals was consistent with the findings of follicular dynamics corroborated by *Ginther* et al. (2007). With the data adjusted using the FCC, we observed that the serum P4 concentration patterns in mares obtained using CLIA was similar to those reported by *Kozicki* et al. (2018) (value < 5.48 ng/mL of serum P4 defined the follicular phase and ≥ 5.48 ng/mL defined the luteal phase of the oestrous cycle). Therefore, it is possible to observe an increase in serum P4 in mares from d3 of the cycle extending to d17 (Tab. 2) when the levels drop, initiating the follicular phase of the oestrous cycle. In view of these data, our initial hypothesis that CLIA methodology is efficient in determining the P4 profiles in the serum of mares during the oestrous cycle is confirmed.

Based on ovarian findings (daily scans), it was possible to establish the follicular and luteal phases of the oestrous cycle in mares. The latter differed in length because there was a variation of 22 and 27 days (Fig. 5 and 6). The length of the oestrous cycle was from 21.2 (lactating) to 22.8 days (non-lactating) (*Aurich* 2011), which varied according to the race and body condition score.

We conclude that CLIA can be used as an alternative method for serum P4 determination in mares. The P4 concentrations determined using CLIA were correlated with the follicular dynamic findings obtained using US examinations and with those obtained using ELISA. Therefore, CLIA is an efficient, reliable, low-cost and rapid method for processing the blood samples to determine the P4 concentration.

Animal Welfare Statement

The project was approved by the CEUA (Pontificia Universidade Catolica do Paraná, Brazil) under protocol 01145.

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