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Development and Characterization of a Homologous Radioimmunoassay for Equine Prolactin (41829)

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Abstract. A specific and sensitive homologous radioimmunoassay has been developed for equine prolactin, suitable for measuring prolactin concentrations in serum of horses. The sensitivity of the assay ranged from 0.4 to 0.6 ng/ml and the intra- and inter-assay coefficients of variation averaged 6.9 and 15.4%, respectively, for five doses of hormone. Cross-reactivity with other mammalian and nonmammalian prolactins and growth hormones was less than 20 and 0.3%, respectively. Cross-reactivity with equine growth hormone was less than 0.07%. Equine serum and pituitary extracts showed parallel dilution–response curves with equine prolactin. The percentage recovery of exogenous equine prolactin in serum was 89%. Preliminary analysis of several physiological samples (stallions, pregnant, and nonpregnant mares) yielded values from 0.6 to 12.0 ng/ml.

The physiological role of prolactin in the horse has not been well defined. Investigations of the mechanisms controlling prolactin synthesis and secretion as well as the physiological function of prolactin in the horse require a sensitive and specific method of measuring this hormone in serum. Recently, the isolation and properties of equine prolactin were reported (1, 2). Chen (1) included a report on the development of a homologous radioimmunoassay (RIA) for equine prolactin (ePRL). The assay, however, was only partially characterized and the antiserum proved to be neither species specific nor highly sensitive. Physiological serum concentrations of ePRL were not investigated.

Only a few studies in the horse have involved measurements of serum prolactin. The investigators employed a homologous RIA for ovine PRL (3) to follow patterns of prolactin secretion in the mare associated with pregnancy (4), postpartuition (5), and melatonin or TRH treatment during the breeding season (6). As reported in these studies, levels of prolactin were either extremely variable or changes were undetectable. Although the homologous RIA for ovine prolactin was reported to be specific for PRL, equine growth hormone (eGH) was not tested for cross-reactivity. The structural differences between ePRL and oPRL as reported by Li and Chung (2) suggest there may be immunological differences that could affect results obtained in the analysis of equine sera. Therefore, a specific homologous radioimmunoassay may be a more reliable tool to study fluctuations of serum PRL concentrations in horses.

The objective of this investigation was to develop and characterize a highly specific and sensitive homologous RIA for equine prolactin, suitable for measuring the concentrations in serum of horses.

Materials and Methods. The preparation of highly purified equine prolactin employed in these studies has been described previously (2). All other preparations, except as noted, were highly purified, prepared in these laboratories, and in most cases have been described in previous publications. These included ovine (o) PRL (7) and GH (8), porcine (p) PRL (9) and GH (8), human (h) GH (8), chorionic sommatomammotropin (CS) (10), equine (e) LH and FSH (11), TSH (12), and canine (d) PRL (13). The rat PRL (14) was obtained from Dr. A. F. Parlow (Torrance, Calif.). The equine GH, canine GH, human PRL, and rat GH were highly purified and partially characterized in these laboratories (unpublished results). Purified nonmammalian hormones included ostrich PRL and GH, sea turtle PRL and GH, and *Tilapia* PRL and GH (teleost) (15).

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The equine pituitary extract (E41E) was an alkaline extract, dialyzed and lyophilized from whole equine pituitaries. The pituitary preparation E234P was a fraction derived from E41E. Human serum albumin (HSA, fraction V), bovine serum albumin (BSA, fraction V), and ethylenediamine tetraacetic acid (EDTA, disodium salt) were purchased from Sigma Chemical Company, St. Louis, Missouri, lactoperoxidase from Calbiochem-Behring Corp., La Jolla, California, and $Na^{125}I$ (carrier-free) from New England Nuclear, Boston, Massachusetts. All other reagents and chemicals were reagent grade.

Preparation of antisera to PRL. Antisera were raised in a mature male New Zealand albino rabbit by the multiple-site injection technique (16). For the initial immunization, 235 μg of purified equine PRL was dissolved in 0.5 ml 0.9% saline, emulsified with 1 ml complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected intradermally into 10 sites over the dorsal surface of the rabbit. The rabbit was injected in a similar fashion 17 days later with 200 μg of ePRL. Blood was collected 10 days after the second injection by marginal ear vein. A titer study indicated the rabbit was responding by producing an antiserum that resulted in 30% specific binding to ^{125}I-ePRL at an initial dilution of 1:1250. Nonspecific binding was 3.0%. Two more injections (100 μg ePRL/injection) were given 1 month apart. A second bleed taken 16 days later had a relatively good titer, binding 30% of the radioligand at an initial dilution of 1:100,000. Analysis of a subsequent bleed taken 2 months later without a prior injection of ePRL indicated that antibody production had declined; an initial dilution of 1:36,000 resulted in 30% specific binding to the ^{125}I-ePRL. A fifth injection (55 μg ePRL), prepared and given in a similar manner, increased the antibody titer back to 1:80,000 after a 2-week time period.

Radioiodination. Highly purified equine prolactin was radiolabeled with $Na^{125}I$ using a modification of the lactoperoxidase method (17). Briefly, 5 μl (1 mCi) of $Na^{125}I$ in 0.01 *M* NaOH were added to a vial containing 10 μg of ePRL in 25 μl of 0.4 *M* sodium acetate, pH 5.6. The iodination reaction was then initiated at room temperature by the addition of 10 μl of a lactoperoxidase solution in 0.4 *M* sodium acetate, pH 5.6. (8.5 μg/10 μl; 0.2 units/10 μl), and three 10-μl aliquots of H_2O_2 (1:30,000 dilution of a 30% solution) at 2-min intervals. The remaining free iodine was eliminated by the addition of 50 μl of a 1 m*M* tyrosine solution. The labeled hormone was separated from the free iodine and iodinated tyrosine by gel filtration on Sephadex G-100 previously coated with 0.5% HSA and eluted with 0.01 *M* phosphosaline buffer, pH 7.5. The specific activity of the ^{125}I-ePRL obtained was 34.7 ± 7.9 μCi/μg (SD, $N = 7$).

Assay conditions. All dilutions were made with 0.01 *M* sodium phosphate buffer, pH 7.4, containing 0.15 *M* NaCl, 0.5% BSA, and 0.01 *M* EDTA. To 0.1 ml of unlabeled prolactin (sample or standard) was added 0.1 ml of rabbit anti-equine prolactin serum and 0.05 ml of ^{125}I-ePRL (10,000 cpm) in 12 × 75-mm borosilicate glass disposable culture tubes (Scientific Products). After an incubation of 16–24 hr at 4°C, 0.1 ml of a solution of sheep anti-rabbit γ-globulin (1:80) and normal rabbit serum (1:200) was added and a second incubation was carried out at 4°C for 16–24 hr. Separation of the precipitated (bound) and free radioactivity was achieved by adding 1 ml of cold PBS and centrifuging the tubes at 1500*g* for 30 min. The supernatant fraction was drained and the tip of each tube dried with cotton swabs. The antibody-bound ^{125}I-ePRL in the precipitate was counted in an automatic γ spectrometer (Beckman Model 4000; 74% efficiency). The displacement of antibody-bound ^{125}I-ePRL by various concentrations of standard ePRL was expressed as a percentage of the ratio $B - NS/B_0 - NS$ where B_0 is the amount of ^{125}I-ePRL bound to antibody in the absence of unlabeled hormone and NS is nonspecifically bound ^{125}I-ePRL.

Validation methods. Specificity. The specificity of the assay was evaluated by comparing the amount of various unlabeled hormones that could inhibit 50% of the binding of ^{125}I-ePRL. A series of mammalian and nonmammalian hormones as well as crude pituitary extracts and horse sera were analyzed as to their parallelism and cross-reactivities.

Recovery. In order to determine if variations in the incubation media had any effect on the binding kinetics of the assay when using serum samples, a test for recovery was performed. Increasing amounts of a purified preparation

of ePRL (0.04–3.3 ng/tube) were added to a constant volume of equine serum with a known concentration of ePRL and analyzed in an RIA. If the variation in incubation media had no effect, linear regression analysis of the amount predicted as the amount measured in terms of the standard should give a slope of 1.0 and intercept of 0.

Immunoaffinity chromatography. The procedures for the preparation of the column and assay protocol as described by Moudgal and Papkoff (19) were slightly modified. The column was prepared in a similar fashion with the exception that rabbit antiserum to ovine prolactin was coupled to the Affigel-10 (Bio-Rad Labs., Richmond, Calif.) and the gel was extensively washed with RIA buffer before use. A 0.5-ml preparation of purified equine prolactin (20 ng/ml) or a 0.5-ml equine serum sample (12 ng/ml equine prolactin as previously determined by RIA) were each applied to the column and run separately. The first 5 ml containing the unabsorbed protein were collected in one tube. Aliquots (0.05 and 0.1 μl) were analyzed for equine prolactin in the RIA.

Equine blood samples. Blood samples were collected from mares during their estrous cycle, pregnancy, and lactation. Samples were also collected from stallions, geldings, and ovariectomized mares. The blood samples were centrifuged and serum stored at −20°C. Samples were assayed for ePRL within 2 months of collection.

Heterologous oPRL RIA. A heterologous oPRL RIA was tested using a purified preparation of oPRL as the radioligand and a 1:3000 dilution of the rabbit anti-ePRL serum. Equine prolactin and oPRL were incorporated as standards. The ePRL concentrations of six horse serum samples were analyzed.

Results. *Precision and reproducibility.* A precision profile (19) based on the intra- and interassay coefficient of variation (CV) for five different doses of hormone is shown in Fig. 1. The average intra- and interassay CVs were 6.9 and 15.4%, respectively.

Specificity and its evaluation. The ability of mammalian and nonmammalian hormones to inhibit 50% of the binding of ^{125}I-ePRL to the antibody was evaluated with reference to the purified ePRL (Figs. 2A and B). Both pPRL and dPRL showed significant inhibition

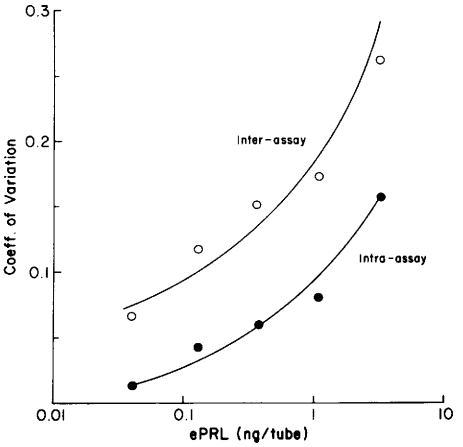

FIG. 1. A precision profile based on the intra- and interassay coefficient of variation for five different doses of purified ePRL. Each point represents the mean of eight determinations derived from eight separate assays.

with cross-reactivities of ~20% but the slopes were nonparallel in comparison to ePRL. Slight inhibition and nonparallelism was observed for hPRL and oPRL. There was no cross-reactivity with rPRL. In the case of growth hormone, eGH, pGH, and dGH showed slight cross-reactivities of 0.07, 0.3, and 0.25%, respectively. Only the eGH showed parallelism. However, oGH, hGH, and rGH at concentrations of 10,000 ng/tube showed no significant displacement. The equine gonadotropins, eLH and eFSH, and eTSH and hCS did not significantly cross-react.

Several nonmammalian hormone preparations were assessed as to their cross-reactivity with ePRL (data not shown). Nonparallel curves demonstrating slight cross-reactivity (<0.01%) were observed with ostrich and sea turtle prolactin. *Tilapia* prolactin did not cross-react. The growth hormones from all three species showed insignificant inhibition (<0.001%).

Sensitivity. The limits of detection for purified ePRL were 0.4–0.6 ng/ml. This could be significantly discriminated from zero at the 95% confidence level (on the basis of twice the standard deviation of the zero value) (Fig. 3). For optimal sensitivity the antiserum was used at an initial dilution of 1:100,000 to obtain 30% binding of the added ^{125}I-ePRL (10,000 cpm) and precipitation was carried out with a double-antibody system.

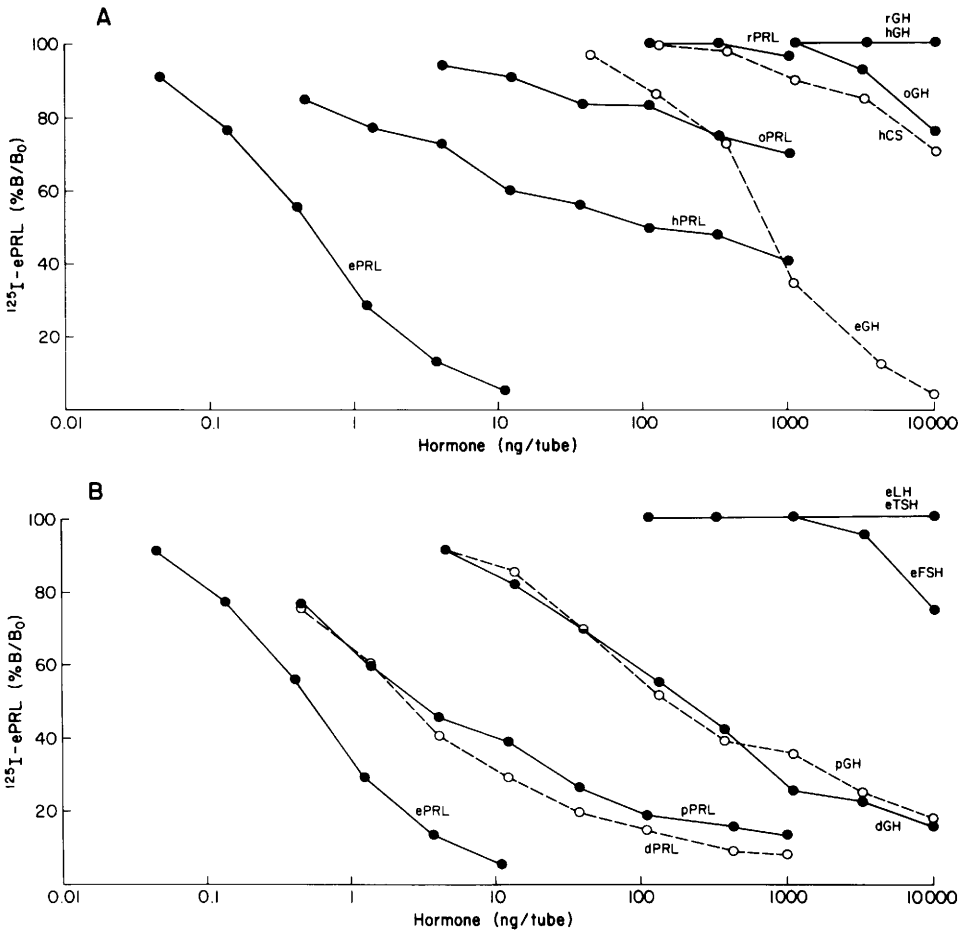

FIG. 2. (A and B) Dose-response curves for purified preparations of various protein hormones in the RIA for ePRL; details of the preparations are given under Materials and Methods.

Immunological characterization of serum and pituitary ePRL. The equine prolactin activities in horse sera and pituitary extracts were characterized by comparing their cross-reactivities with that of the equine prolactin standard. Shown in Fig. 3 is the inhibition of binding by two crude preparations derived from equine pituitaries (E41E and E234P) and a high and low equine serum sample collected from a mare and stallion, respectively. After logit transformation, regression analysis of the inhibition curves revealed that the slopes of all preparations were parallel (not significantly different within a 95% confidence limit) to the purified ePRL standard.

Percentage recovery. The quantitative recovery of known amounts of ePRL from equine serum is shown in Fig. 4. The regression equation was $Y = 0.9297x - 0.0777$. The average percentage recovery was 89%.

Immunoaffinity chromatography. As an additional indication that the displacement activity seen in the horse serum samples was associated with prolactin, a high titer PRL serum sample and a purified preparation of ePRL were independently passed through an oPRL antibody affinity column. An RIA analysis of the eluate indicated that 72–82% of prolactin activity in the horse serum and standard, respectively, remained bound to the affinity column.

Serum concentration of ePRL in horses during various reproductive states. Outlined in Table I is a summary of serum concentrations

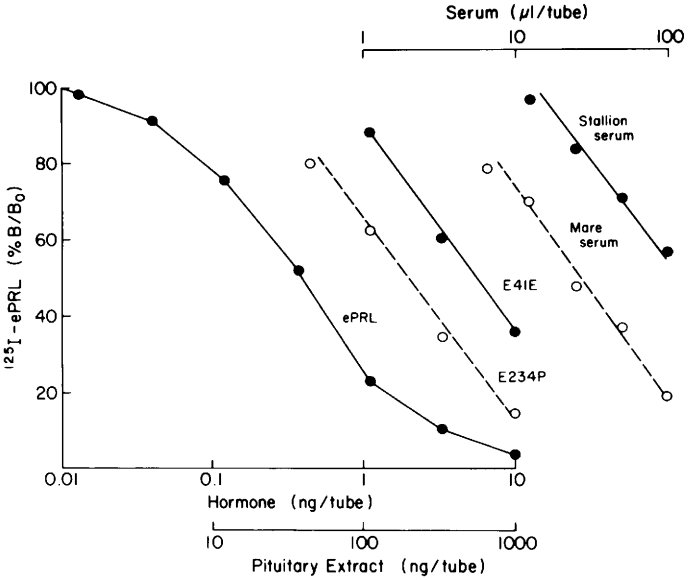

FIG. 3. Inhibition curves for the ePRL reference standard, ePRL in two equine pituitary extracts and ePRL in two samples of serum from a stallion and a mare; demonstrating parallelism.

of ePRL taken from horses in various reproductive states. Serum concentrations ranged from 0.44 to 12.00 ng/ml with the lowest found in a mare at 2 days preparturition and the highest found in a mare 282 days pregnant. Statistical analysis was not performed since in some groups the number of animals was only one.

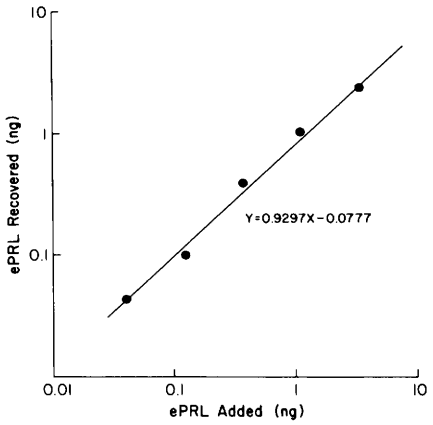

FIG. 4. Recovery of ePRL from equine serum. Each point represents the amount of ePRL recovered from a serum sample assayed in duplicate and corrected for endogenous prolactin. The average percentage recovery was 89%.

Comparison of the homologous ePRL RIA with a heterologous ovine prolactin RIA. In the heterologous oPRL RIA (^{125}I-oPRL–ePRL ABY) the oPRL standard was more sensitive at the lower doses than the ePRL standard. However, the shape and slope of the two curves were not similar (Fig. 5). As indicated in Table II, the serum concentrations of ePRL analyzed in the heterologous oPRL RIA were about two to ninefold higher, using either an ePRL or oPRL standard, than in the homologous ePRL RIA. The range at the lower levels in the heterologous assay was 2.8–8.4 compared to 0.6–5.0 in the homologous assay.

Discussion. Although a few studies on the secretion of equine PRL under various physiological conditions have been reported (5, 6), these studies were based on a homologous ovine PRL radioimmunoassay. Radioimmunoassays performed with material derived from a species different than the one being analyzed may not be sufficiently sensitive to detect circulating levels of hormone (20). With the availability of a highly purified equine prolactin (2) for radiolabeling and for antibody production, a sensitive homologous radioimmunoassay for equine prolactin has been developed which appears suitable for various kinds of physiological studies in the horse.

TABLE I. PROLACTIN LEVELS IN SERUM FROM HORSES IN DIFFERENT REPRODUCTIVE STATES

Horse No.	Reproductive state	Number of animals	Prolactin (ng/ml)	
			Mean	Range
1	Day of ovulation	1	2.85	
2	8th Day after ovulation	1	3.85	
3	282 Days pregnant	1	12.00	
4–6	2–11 Days preparturition	3	1.17	0.44–2.28
7–8	3 Days postparturition	2	4.55	4.10–5.00
9	60 Days postparturition	1	3.30	
10	90 Days postparturition	2	4.14	4.08–4.20
12–15	Spayed mares	4	2.23	0.55–3.70
16–21	Stallions	6	2.19	1.24–3.25
22	Gelding	1	1.25	

The precision profile of the assay indicates a high degree of reproducability as inferred from the close parallelism of the inter- and intrassay CV patterns (Fig. 1).

Cross-reaction studies with purified preparations of several mammalian and nonmammalian prolactins and growth hormones as well as equine glycoproteins showed that the assay was highly specific for ePRL (Figs. 2A and B). With respect to its structure, ePRL has only four half-cystine residues (as do some nonmammalian prolactins) in contrast to other mammalian prolactins which have six residues (2). The circular dichroism spectra indicates that ePRL's secondary structure is different than other mammalian prolactins, particularly ovine prolactin (2). These structural differences may have effected the immunoactivity of ePRL with respect to ovine, rat, and human prolactin as seen in our studies (Fig. 2A). The fact that dog and porcine prolactin cross-reacted somewhat significantly (~20%) in a nonparallel manner to ePRL may be a function of similar antigenic determinants located on the outer surface of the molecules. The nonmammalian hormones do not appear to have similar antigenic sites in reference to ePRL.

In the case of equine growth hormone it is noteworthy that its cross-reactivity in reference to ePRL is <0.07% (Fig. 2A) suggesting very little cross-contamination with ePRL. Since the primary structure and function between growth hormones and prolactins are similar,

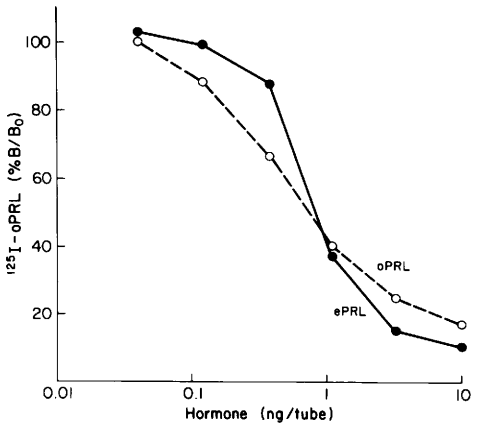

FIG. 5. Inhibition curves for equine and ovine prolactin standards in a heterologous oPRL RIA employing ^{125}I-oPRL and a rabbit anti-equine prolactin serum (1:3000 dilutions = 30% B/T). Each point represents the mean of triplicate determinations.

TABLE II. COMPARISON OF ePRL SERUM CONCENTRATIONS ANALYZED IN A HETEROLOGOUS oPRL RIA AND A HOMOLOGOUS ePRL RIA

Horse No.	Prolactin (ng/ml)		
	Heterologous oPRL RIA		Homologous ePRL RIA
	ePRL Std.	oPRL Std.	ePRL Std.
22	5.2	2.8	1.3
12	7.0	5.3	2.7
15	4.8	2.4	0.6
8	8.2	7.1	5.0
11	8.4	7.4	4.1
3	28.5	110.0	12.0

in some mammalian species (21, 22), it is important that prolactin activity in eGH is low for validation of this assay. No interference by serum albumin or other serum components was observed in the RIA as demonstrated by the parallelism of the inhibition curves of serum prolactin and crude PRL pituitary extracts (Fig. 3) and excellent recoveries of exogenous ePRL added to serum samples (Fig. 4).

The immunoaffinity chromatography study provides additional evidence that the immunoactivity seen in horse serum in the RIA is indeed prolactin and not an interferring substance.

The data on serum prolactin levels in horses taken during various reproductive states is not complete. Ongoing experiments investigating physiological patterns in more detail will be reported in the future. However, the present data (Table I) suggests that in the mare, levels are highest during pregnancy and lowest during prepartuition. Nett *et al.* (4) found prolactin levels to be quite variable in mares during gestation. It also appears that stallions and spayed mares have similar serum prolactin levels (Table I).

Because of the unavailability of highly purified, species specific hormones, heterologous RIAs are generally employed to determine serum or plasma hormone concentrations. It was of interest to compare the equine PRL serum concentrations derived from a heterologous ovine prolactin RIA vs a homologous equine prolactin RIA. As indicated in Fig. 5, the shape and slopes of ePRL and oPRL in the heterologous assay are quite different which appears to have a significant effect on the serum prolactin results (Table II). The wider range at the lower levels indicates that the homologous ePRL RIA is a much more sensitive assay for equine prolactin levels. The flat, nonparallel slope of the oPRL standard in the homologous ePRL RIA (Fig. 2A) suggests that the binding affinity of the hormone to its antibody can differ considerably depending on the preparations used.

In conclusion, the availability of a sensitive and highly specific ePRL RIA will allow for future meaningful investigations of the regulation of synthesis and secretion of prolactin and the physiological role of this hormone in the horse.

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