The full details of the published version of the article are as follows:

Comparison of immunofluorescence and chemiluminescence assays for measuring ACTH in equine plasma

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Summary

Background: The chemiluminescence (CL) and immunofluorescence (IF) assays yield different results for basal adrenocorticotropin hormone concentrations [ACTH] in pony plasma. It is unclear whether this difference also occurs in basal samples from horses or samples from ponies following thyrotropin-releasing hormone (TRH) stimulation.

Objectives: To compare the results of [ACTH] analysis by CL and IF methods in basal samples from horses and pony samples following TRH stimulation.

Study design: Method comparison.

Methods: Plasma [ACTH] was measured concurrently using CL and IF methods in 12 ponies (basal and post TRH stimulation) in November and basal samples from horses (n = 45; November and May).

Results: CL and IF methods yielded different results (P<0.01). The median difference (CL – IF) (95%CI) for ponies was 5.9 (0.1-7.5)pg/ml at baseline and 227.9 (61-1001)pg/ml post TRH; and horses 1.9 (1.1-5.4)pg/ml in November and 9.4 (8.2-11.5)pg/ml in May, at baseline. Correlation was good in ponies at baseline (R=0.80, p=0.003) but not post TRH, and good in horses in November and May (R=0.68 and 0.71, p<0.001). Bland-Altman analysis demonstrated moderate bias and wide 95% limits of agreement (95%LOA) in ponies at baseline (bias 5.5pg/ml; 95%LOA -9.9-20.9pg/ml) and horses in May (bias 10.6pg/ml; 95%LOA -9-30.3pg/ml) and very large bias and wide 95%LOA in ponies post TRH (bias 477pg/ml; 95%LOA -633-1587pg/ml). Using CL cut-offs of >29pg/ml and >110pg/ml, agreement was moderate (k=0.67) and very good (k=0.82) for binary classification of PPID in ponies at baseline and post TRH; and good (k=0.73) for horses in November, but poor (k=0.40) in May.

Main limitations: Limited numbers of horses with [ACTH] above threshold values.

Conclusions: The assays yielded different absolute values, particularly in post TRH samples from ponies, suggesting TRH stimulates secretion of cross-reacting peptides other than ACTH. Agreement for binary classification for PPID was moderate to good, except in basal samples from horses in May.

Introduction

Analysis of plasma adrenocorticotropin hormone concentration [ACTH], both at baseline and following thyrotropin-releasing hormone (TRH) stimulation, is used to aid the diagnosis of equine pituitary pars intermedia dysfunction (PPID) [1]. The TRH stimulation test can be used to assist diagnosis of PPID where baseline [ACTH] is equivocal or negative and PPID is suspected. Plasma [ACTH] can vary in individuals and TRH stimulation tests are thought to reduce the relative impact of confounding factors by maximally and specifically stimulating the pars intermedia of the pituitary gland [2]. Two methods for measurement of [ACTH] in the horse, namely the chemiluminescent (CL) and immunofluorescent (IF) assays, have demonstrated acceptable precision and accuracy [3]. Seasonally adjusted CL reference ranges for baseline [ACTH] are widely used, and more recently seasonally adjusted post TRH stimulation [ACTH] cut-offs have been reported [4,5]. The post TRH stimulation test [ACTH] cut-off values are an ongoing area of research, with current cut-offs indicating a wide range of equivocal values [6].

It was previously demonstrated that the CL and IF methods yielded significantly different results when [ACTH] was measured in EDTA plasma samples collected from ponies in spring and autumn. In addition, the association between these CL and IF results were shown to differ in autumn and spring, consistent with seasonally dependent assay cross-reactivity or interference [7]. Corticotropin-like intermediate lobe peptide (CLIP) was considered to be the most likely in vivo cross reactant [7]. Previous studies have demonstrated endocrine differences between horses and ponies including differences in concentrations of α-MSH, ACTH and insulin [8,9]. Thus, it is possible that pituitary-derived cross reactants may have different effects with respect to the measurement of [ACTH] using these two assays in plasma samples collected from horses rather than ponies. In addition, the possibility of pituitary-derived cross-reactants affecting the measurement of [ACTH] in samples collected from ponies following TRH stimulation has yet to be determined. Thus, the aim of the current study was to compare [ACTH] as determined by the CL and IF methods in baseline plasma samples collected from horses and ponies and in samples from ponies following TRH stimulation.

Methods

Twelve university kept ponies and 90 clinically healthy, mature adult, non-Thoroughbred horses, kept as a single group with one owner, were included in the study. The ponies were all British native breed mares and <148cm in height at the withers with a mean age of 17.42 (range: 9 – 25) years. Seven of the ponies had a history of previous laminitis and two had hypertrichosis. All ponies underwent TRH stimulation tests on the same day in late November following a previous equivocal baseline [ACTH]. The horses were all ≥148cm in height and 67 were geldings and 23 were mares. None of the horses had any clinical signs consistent with PPID. Residual plasma was obtained following blood sampling for tapeworm infection monitoring on one of two dates in November (n=45) and in May (n=45). All animals were clinically healthy at the time of sampling and none were pregnant or lactating.

In the ponies, a baseline blood sample was obtained from the jugular vein into plastic EDTA vacutainers[a]. This was followed by intravenous injection of 1 mg TRH[b]. A second EDTA blood sample was collected after 10 minutes. Horses' residual blood was put into plastic EDTA vacutainers[a] following sampling from the jugular vein. All whole blood samples were immediately chilled on ice, separated by centrifuge (10 min at 2000x g) within 6 hours and the plasma aliquoted and frozen at -80°C on the same day until analysis. Samples were thawed and analysed on the same day. Concurrent analysis of each sample was performed on two automated analysers: a solid phase 2-site sequential chemiluminescent immunometric assay using mouse monoclonal and rabbit polyclonal anti-human ACTH antibodies (ACTH Immulite 1000[c]) and a 2-site immunoenzymometric assay using 2 types of polyclonal goat anti-human ACTH antibodies (ST AIA ACTH[d]). Analysis and quality controls were performed according to manufacturers' instructions.

Data analysis

Animals with ACTH concentrations above the reference interval, according to published seasonally adjusted [ACTH] cut-offs [4,6], were reported as percentages for each sample group. The median and interquartile ranges (IQRs) were calculated for the results of each method among ponies at baseline and following TRH stimulation and among horses at baseline in November and May. The distributions of the differences between paired ACTH values (CL – IF) were analysed for normality using histograms and Shapiro-Wilk normality tests. To determine whether there was a difference between the results obtained from the CL and IF assays the [ACTH] of the concurrently analysed samples were compared using Wilcoxon matched-pairs signed rank tests. The relationships between the CL and IF [ACTH] were examined using scatter plots, Spearman correlation and linear regression. The residuals of linear regression were normally distributed. The slopes of the linear regression curves compared using a F tests. Absolute difference Bland-Altman (Bland-Altman) plots were used to examine the bias and 95% limits of agreement (LOA) between the methods among ponies at baseline and following TRH stimulation and among horses at baseline in November and May.

Samples with CL [ACTH] > 29pg/ml at baseline and >110pg/ml following TRH stimulation were classified as positive for PPID and samples with [ACTH] below these values were classified as negative [4,6]. ROC analysis and the maximum Youden index method were used to determine corresponding IF cut-offs to the CL cut-offs. Kappa analysis was used to determine agreement between the assays for binary classification. Kappa values of >0.81 were considered to indicate very good agreement, 0.61-0.8 good, 0.41-0.6 moderate, 0.21-0.4 fair and <0.2 as poor [10].

Data were processed, and analyses performed using spreadsheets (Microsoft Excel[e]) and statistical software[f].

Results

Two samples from horses in November yielded CL [ACTH] below the lower limit of detection and were thus excluded from further analysis. The number of ponies with ACTH concentrations above the reference values was 4/12 using baseline values and 8/12 using values following TRH stimulation; all 4 ponies with ACTH concentrations above the reference values at baseline also had results above the reference value following TRH stimulation. Two ponies with previous laminitis and one with hypertrichosis had ACTH concentrations above the reference values at baseline. Both ponies with hypertrichosis and 5 ponies with previous laminitis had ACTH concentrations above the reference value following TRH stimulation. In horses, 12% (5/43) had ACTH concentrations above the reference value in November and 22% (10/45) in May.

The median (IQR) [ACTH] (pg/ml) values measured using the CL and IF assays were 20.2 (29) and 19.2 (26.8) in ponies at baseline (Fig 1); and 274 (851.3) and 43.7 (63.3) in ponies following TRH stimulation (Fig 2), respectively. In horses, this was 17.5 (4.5) and 15.7 (7.6) in November and 24.6 (8.3) and 13.4 (8.4) in May (Fig 3).

The median difference between assay results (CL – IF) (95%CI) were 5.9 (0.1-7.5) pg/ml (p=0.01) at baseline and 227.9 (61-1001) pg/ml post TRH (p<0.001) in ponies; in horses they were 1.9 (1.1-5.4) pg/ml in November (p=0.003) and 9.4 (8.2-11.5) pg/ml in May (p<0.001).

Spearman correlation between assays in ponies was good at baseline (R = 0.80, p=0.003) but weak post TRH (R = 0.57, p=0.06). Among horses, Spearman correlation was good (R = 0.68 and 0.71, P<0.001) in November and May, respectively.

Linear regression demonstrated IF = 0.68 X CL + 3.94 (R^2 = 0.90) in ponies at baseline (Fig 4), IF = 0.62 X CL + 4.41 (R^2 = 0.47) in horses in November and IF = 0.53 X CL + 1.8 (R^2 = 0.46) in horses in May (Fig 5). There was no evidence for a significant difference (F=0.27; p=0.6) when comparing the linear regression slopes of the horses in November and May.

Bland-Altman analysis (Fig 6) demonstrated mean bias and 95% limits of agreement (95% LOA) of 5.52pg/ml (-9.91 - 20.94pg/ml) in ponies at baseline and 477pg/ml (-633.20 - 1587pg/ml) in ponies following TRH stimulation. In horses they were 10.61pg/ml (-9.04 - 30.25pg/ml) in May and 0.037 pg/ml (-38.49 – 38.56pg/ml) in November.

Receiver operating characteristic (ROC) and Youden index calculations, using a CL non-autumn cut-off of 29pg/ml and post TRH cut-off of 110pg/ml, demonstrated the greatest agreement between IF and CL assays for binary classification when a diagnostic cut-off of >22.90pg/ml was used for the IF assay for baseline ACTH concentrations in November and >15.2pg/ml in May for horses, and >19.2pg/ml for baseline ACTH concentrations and > 32.2pg/ml for ACTH concentrations following TRH stimulation in ponies in November.

Using the same CL assay cut-offs there was moderate (ƙ=0.67) and very good (ƙ =0.82) agreement for binary classification of PPID in ponies at baseline and post TRH. In horse agreement was good (ƙ =0.73) in November, but poor (ƙ =0.40) in May.

Discussion

The assay used to measure ACTH concentration is important for the clinical interpretation of the results. Poor agreement between equine [ACTH] values measured using CL methods and a commercial radioimmunoassay has been reported previously [11]. The findings of this study agree with those of Irvine *et al*. [3] and Knowles *et al*. [7], demonstrating that the [ACTH] is also significantly lower when measured using IF compared to CL methods in concurrently run samples obtained from horses or ponies at baseline or ponies post TRH stimulation test.

The differences between the assay results in this study were large enough to affect clinical interpretation and highlights the requirement for assay specific thresholds. The correlation of [ACTH] measured in ponies using the two assays was good at baseline, however the median of differences, the Bland-Altman bias and 95% limits of agreement (LOA) were deemed large and wide enough to impact clinical interpretation of results. The post TRH stimulation test results were not significantly correlated, the median of differences was very large and Bland-Altman analysis

demonstrated very large bias and wide 95% LOA. Together, these findings indicate that the absolute test results were not comparable at baseline nor following TRH stimulation in ponies. However, due to the small sample size, the wide LOA and the magnitude of the differences between assays in this group, this should be interpreted with caution. There was a statistically significant difference in results using samples from horses obtained at baseline in both November and May. The differences seen in November were very small and unlikely to affect clinical interpretation using CL diagnostic thresholds, with low Bland-Altman bias and the wider 95% LOA likely influenced by a single outlier. In May, the median of differences was deemed large enough to affect interpretation and the Bland-Altman bias and 95% LOA were also large. Thus, overall the tests were comparable in November but not May. Knowles *et al.* [7] identified a different relationship between [ACTH] generated by the two methods when comparing samples collected from the same ponies in the autumn and the spring. In the present study the association between the [ACTH] results in horses did not differ significantly with time of year, however both sampling times in the current study were classified as non-autumnal, and this result is therefore not unexpected.

The most notable finding of the current study is the large difference in [ACTH] between the two assays following TRH stimulation. TRH receptors on the melanotrophs of the pars intermedia are stimulated by exogenous TRH, leading to secretion of α-MSH, CLIP, and β-endorphin, with hyperplastic or neoplastic melanotrophs also secreting significant amounts of ACTH [12]. Our finding in ponies of very large differences in [ACTH] following TRH stimulation supports the theory set out by Knowles *et al*. [7] of probable cross-reactivity or interference by another substance in the CL assay, resulting in the large differences in [ACTH] between the two methods. Given the reported cross reactivity of the CL assay to synthetic CLIP in equine plasma [7], CLIP would be a likely candidate peptide for the effect seen in the present study following TRH stimulation. The cross reactivity likely occurs due to the antibody targets. For the IF assay, the targets are ACTH 1-16 and 24-39 and so the assay will only detect the intact ACTH peptide. Whereas, the primary antibody in the CL assay is directed to CLIP (ACTH 18-39) and the secondary is a polyclonal for ACTH 1-24, so if CLIP is bound to the primary antibody then the N-terminal 7 amino acids of CLIP (ACTH 18-24) can act as an epitope for the CL secondary antibody. ACTH 18-24 is conserved perfectly between horse [13] and human [14] and so the IF assay is therefore likely to be more specific for ACTH. Given the magnitude of the differences between the assay results and degree of reported cross reactivity, it appears that ACTH is a relatively minor product of the pars intermedia in some ponies when compared with the cross reacting POMC(s). Prohormone convertase 2 cleaves ACTH into alpha MSH and CLIP and would be expected to produce equimolar amounts of each, although CLIP may undergo further cleavage [15]. As sampling occurred in late November, consideration must be given to the possibility of late autumnal seasonal effects on the pituitary secretome at this time of year [14].

A non-autumnal [ACTH] cut-off of 29pg/ml [4] and post TRH stimulation [ACTH] cut-off 110pg/ml [6] were used for the classification of samples as above or below the reference values in order to explore binary agreement between the assays. The most recent recommendations outline a 3-tier classification of PPID results with negative, equivocal and positive ranges [6,16]. This classification was not used in the current study due to low numbers falling within the

equivocal ranges and the aim of this study being to compare the assay results, rather than validate or define cut-offs for an assay. These results must therefore be interpreted with caution due to low numbers, particularly among the ponies, and the lack of inclusion of horses with clinical signs consistent with PPID. In addition, these IF cut-off values should not be extrapolated to other populations. The agreement for binary interpretation of the results using the above cut-off values were moderate and very good among ponies pre and post TRH stimulation, and good and poor for horses in November and May, respectively. The poor agreement among horses in May could be explained by a very large number of results being close to the diagnostic threshold.

In conclusion the assays yielded different absolute values, particularly in samples from ponies' post TRH stimulation, suggesting TRH stimulates secretion of cross-reacting peptides other than ACTH. The CL and IF assays cannot be used interchangeably, particularly following TRH stimulation for diagnosis of PPID, and assay specific thresholds are vital. Further work investigating which peptides may be cross-reacting or interfering with the CL assay is warranted.

Authorship

T. McGilvray, N. Menzies-Gow, E. Knowles, and P. Harris contributed to the study design. T. McGilvray and N. Menzies-Gow contributed to the study execution. T. McGilvray, N. Menzies-Gow and E. Knowles contributed to the data analysis and interpretation. McGilvray prepared the manuscript with contributions from N. Menzies-Gow, E. Knowles and P. Harris. All authors approved the final version of the manuscript.

Manufacturers' addresses

[i]BD Vacutainers, Beckton Dickinson, Oxford, UK.

[ii]Phoenix Pharmaceuticals Inc., Karlsruhe, Germany.

[iii]ACTH Immulite 1000, Siemens Healthcare, Sudbury, Suffolk, UK.

[iv]ST AIA ACTH, Tosoh Bioscience, Redditch, Worcestershire, UK.

[v]Microsoft, Redmond, Washington, USA.

[vi]GraphPad Prism 8, San Diego, California, USA.

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Figure legends

Fig 1: Box and whisker plots of [ACTH] measured with the CL and IF assays in ponies at baseline. Whiskers demonstrate the maximum and minimum values; box borders the first and third quartiles and the horizontal line within each box the median. *p=0.01
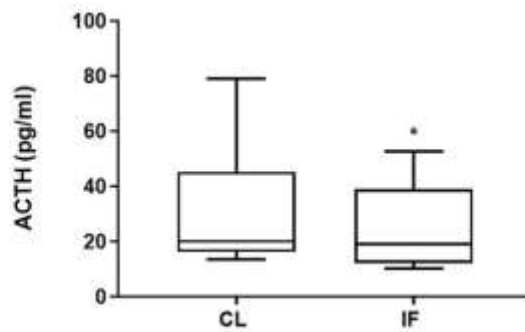

Fig 2: Box and whisker plots of [ACTH] measured with the CL and IF assays in ponies following TRH stimulation. Whiskers demonstrate the maximum and minimum values; box borders the first and third quartiles and the horizontal line within each box the median. *p<0.001
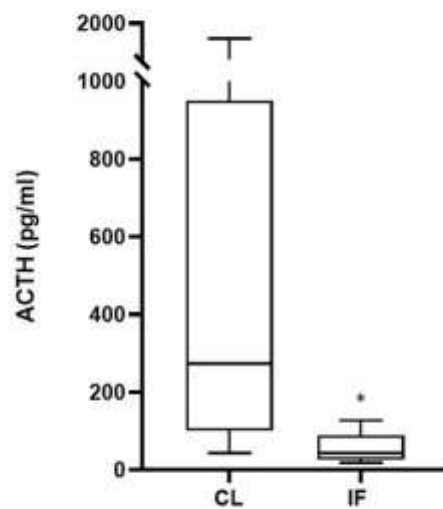
Fig 3: Box and whisker plots of [ACTH] measured with the CL and IF assays in horses at baseline in November and May. Whiskers demonstrate the maximum and minimum values; box borders the first and third quartiles and the horizontal line within each box the median. *p=0.003, **p<0.001
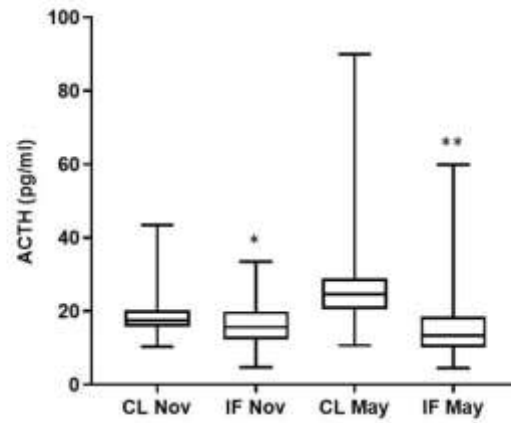
Fig 4: Scatter plots of [ACTH] measured using the CL and IF assays in ponies at baseline with linear regression line fitted. IF = 0.68 X CL + 3.94 (R^2 = 0.90)
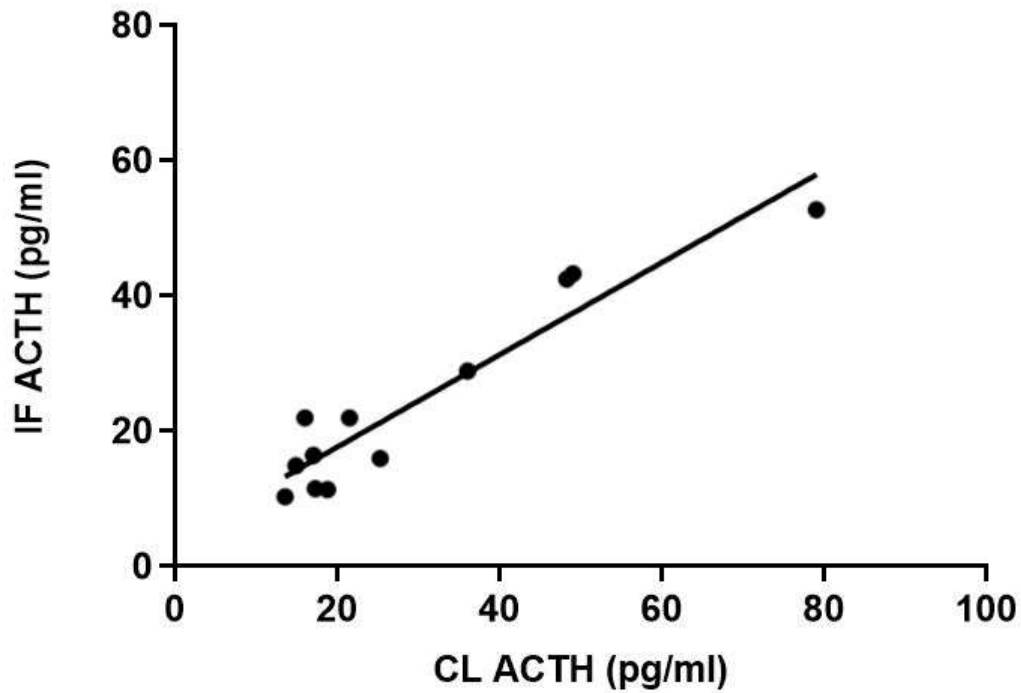
Fig 5: Scatter plots of [ACTH] measured using the CL and IF assays in horses in November and May with linear regression lines fitted. November: IF = 0.62 X CL + 4.41 (R^2 = 0.47), May: IF = 0.53 X CL + 1.8 (R^2 = 0.46)
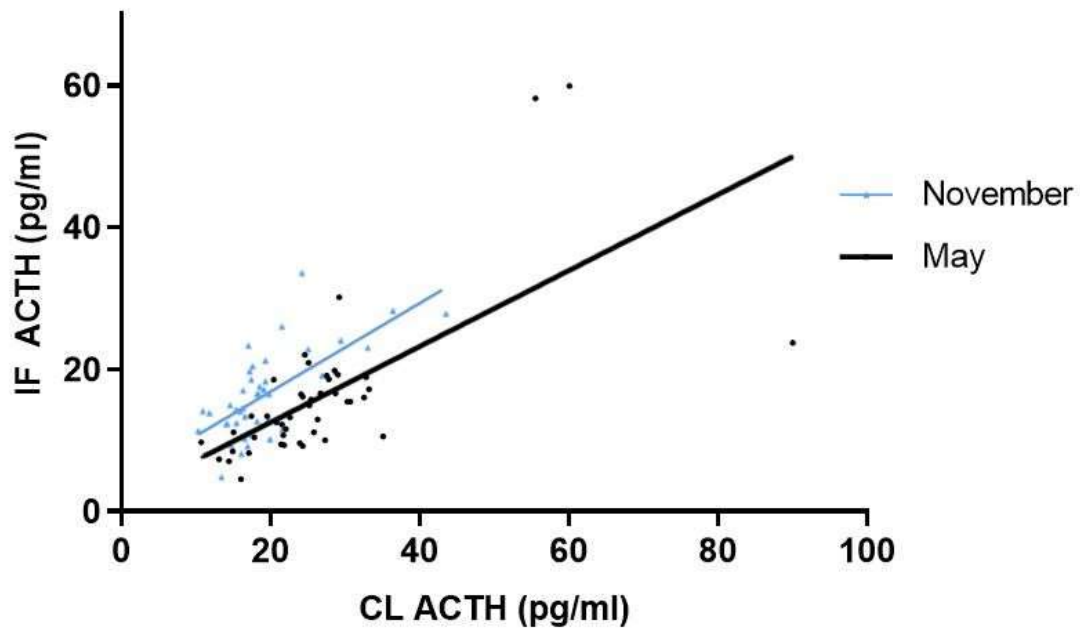
Fig 6: Absolute difference Bland-Altman plots (CL - IF) showing differences between results in: A. ponies at baseline; B. ponies post TRH stimulation; C. horses in May; and D. horses in November. Solid lines represent mean differences and dotted lines the LOA. The mean difference for figure D is 0.04 with the solid line not represented on the chart.