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Research article

Comparison of AlphaLISA and RIA assays for measurement of wool cortisol concentrations

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ABSTRACT

Radioimmunoassay (RIA) methods have always represented a technique of choice for the determination of steroids in biological samples. The Amplified Luminescent Proximity Homogenous Assay-Linked Immunosorbent Assay (AlphaLISA) is now emerging as the new-generation immunoassay technology that does not require washing/separation steps. The aim of this study was to adapt the Perkin-Elmer's AlphaLISA kit for wool cortisol and compare it with a RIA wool cortisol assay. Wool from lambs, 35 at birth (A0) and 54 at two months old (A2), was collected and each extract was evaluated for wool cortisol concentrations (HCC) both by RIA and AlphaLISA immunoassay. The two methods showed good precision, sensitivity and specificity for determining HCC. Both methods were able to detect significant differences between the high and the low HCC assessed in lambs at A0 and A2 ($P < 0.01$). The HCC assessed with RIA were significantly higher than those assessed with AlphaLISA ($P < 0.01$). Moreover, the correlation between HCC measured using the AlphaLISA and RIA methods was strong ($r = 0.878$). The regression analyses show a constant and not proportional error. This could be due to the diversity in the dosage steps and to the diversity of the molecules used in the two methods. Results support the validity of using AlphaLISA as an alternative method to RIA for the quantification of cortisol in sheep wool and considering the performances showed it has a great potential to be further applied as an excellent tool to evaluate HCC in samples derived from animal species.

1. Introduction

Cortisol has been suggested as a marker of the hypothalamic–pituitary–adrenal (HPA) axis activity (Stephens and Wand, 2012) and among the biological matrices in which cortisol can be analysed there is worldwide a high interest toward the utilization of hair as sample. Hair analyses has been successfully used in forensic, toxicological and doping research (Villain et al., 2004) on human samples and on a wide range of animal species to evaluate the allostatic load (Dettmer et al., 2012; Caslini et al., 2016; Biancucci et al., 2016; Crill et al., 2019), environmental conditions (Peric et al., 2017a) and the adaptation to environmental or physiological changes (Peric et al., 2017b) while wool produced by sheep has received relatively little attention (Salaberger et al., 2016; Fürtbauer et al., 2019; Sawyer et al., 2019).

The radioimmunossay (RIA) cortisol determination in hair is largely used in many animal species and primates (Comin et al., 2012; Galuppi et al., 2013; Qin et al., 2015; Massey et al., 2016). RIA methods have always represented a technique of choice for the determination of steroids in biological samples, and to date they are considered to be a reference if other immunometric dosages are to be introduced (Ferraro et al., 2002). Indeed, the RIA assay is the traditional gold standard method for immunoassays (Reimers et al., 1981) as it is sensitive, specific, and reproducible. For some disadvantages of RIA assay related to the use of radioactivity alternative methods have been developed in recent years such as High Performance Liquid Chromatography (HPLC), Fluorometric, Chemilumiscent, Enzyme and Amplified Luminescent Proximity Homogenous Assay-Linked Immunosorbent Assay (AlphaLISA) immunoassay. The AlphaLISA is now emerging as the new-generation immunoassay technology. Homogeneous cortisol AlphaLISA assay reduces hands-on and total assay times and eliminates the need for multiple washes to separate bound from unbound assay components (Beaudet et al., 2008).

The RIA method based on binding of ^{3}H-steroid by competitive adsorption is diffusely used for hair cortisol quantification (González-de-la-Vara et al., 2011; Bacci et al., 2014; Galuppi et al., 2013; Siniscalchi et al., 2013) as well the Enzyme Immune Assay (EIA)/Enzyme

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Linked ImmunSsorbent Assay (ELISA) technique (Macbeth et al., 2010; Fairbanks et al., 2011; Terwissen et al., 2013) that instead of the radioactive isotopes uses a colour reaction for detection of antigen–antibody interactions (Boguszewska et al., 2019). RIA and ELISA methods are sensible and less expensive than mass spectrometry techniques (Sakamoto et al., 2018). Moreover, the coefficient of variation is generally below 10%, which is similar to the coefficients found with High Performance Liquid Chromatography with Mass Spectrometry (HPLC/MS) (Gerber et al., 2012).

The new AlphaLISA bead-based technology relies on Perkin-Elmer's exclusive amplified luminescent proximity homogeneous assay (AlphaScreen®) and uses a luminescent oxygen-channelling chemistry (Beaudet et al., 2008). The commercial cortisol AlphaLISA kit is a competitive immunoassay, in which a Biotinylated analogue of the analyte of interest, the tracer, binds to the Streptavidin-coated Alpha Donor beads, while the Anti-Analyte Antibody is conjugated to AlphaLISA Acceptor beads. In the presence of low analyte, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm. In the presence of high analyte, the beads are separated resulting in lower emission. The advantage of the cortisol AlphaLISA assay format is that it does not require washing or separation steps. This technique has been validated by the manufacturer for human cortisol in serum and plasma, but not for hair or wool cortisol. A lower number of assay steps makes the analyses less time consuming and reduces the possibility of human errors by simplifying the workflow.

The hypothesis tested were: firstly, to adapt and validate the Perkin-Elmer's AlphaLISA kit to assess wool cortisol; secondly, compare the AlphaLISA with a RIA wool cortisol assay. These hypotheses would support the validity of using AlphaLISA as an alternative method to RIA for the quantification of cortisol in sheep wool, a sample that requests high sensitivity of the analytical method and the capability to measure low hormone concentrations.

2. Materials and methods

2.1. Wool sampling, washing and extraction

The wool was collected from 89 male lambs (*Ovis aries*) belonging to the Jezersko-Solčava breed, 35 at birth (A0) and 54 at two months old (A2). All the samples at A2 were re-growth. Electronic clippers were used to shave the wool close to the skin and the samples were stored in paper envelopes in the dark and at room temperature for two months until being processed. Although wool sampling is a non-invasive and non-troublesome procedure, the trial was carried out in accordance with EU Directive 2010/63/EU, and Italian legislation on animal care (DL n. 26, 04/03/2014) and approved by the Ethical committee of the University of Nova Gorica. Wool samples were mechanically cleaned and placed in polypropylene tubes, covered with isopropanol (Merck KGaA, Darmstadt, Germany) (5 mL) and gently mixed for 3 min at room temperature. The sample was again washed with isopropanol and air dried. This washing procedure minimize the risk of extracting cortisol from outside the wool and it also ensure the removal of dust, lanolin and any steroids on the surface of the wool sample due to sweat and sebum. Subsequently, wool was mixed uniformly, placed in a glass vial (60 mg for the RIA analyses and 120 mg for the AlphaLISA immunoassay) with 3 ml of ≥99.8% methanol (Merck KGaA, Darmstadt, Germany) and incubated at 37 °C for 16 h for steroid extraction. After centrifugation at 1000 rpm for 15 min, the methanol in the vial was evaporated to dryness at 37 °C under an air-stream suction hood. The remaining residue was dissolved in 0.6 ml of RIA buffer (0.05 M phosphate-buffered saline, pH 7.5, 0.1% BSA) or in 0.5 ml of AlphaLISA ImmunoAssay Buffer for RIA and AlphaLISA immunoassays, respectively.

2.2. Wool cortisol analyses by RIA method

The wool cortisol from lambs evaluated by the RIA method was measured using a solid-phase microtitre RIA procedure that was already validated in bovine (Peric et al., 2013), horse (Montillo et al., 2014), rabbit (Peric et al., 2017a), ibex (Prandi et al., 2018), pig (Bergamin et al., 2019), red deer (Caslini et al., 2016), dog (Veronesi et al., 2015) and goat (Battini et al., 2015) hair. In brief, a 96-well microtitre plate (OptiPlate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum, diluted 1:1,000 in 0.15 mM sodium acetate buffer, pH 9, and incubated overnight at 4 °C. The plate was washed twice with RIA buffer, pH 7.5, and incubated overnight at 4 °C with 200 μl of the anti-cortisol serum diluted 1:20,000 (Analytical Antibodies, Bologna, Italy). The cross-reactivities of this antibody with other steroids are as follows: cortisol 100%, cortisone 4.3%, corticosterone 2.8%, 11-deoxycorticosterone 0.7%, 17-hydroxyprogesterone 0.6%, dexamethasone 0.1%, progesterone, 17-hydroxypregnenolone, DHEAS, androsterone sulphate and pregnenolone <0.01%. After washing the plate with RIA buffer, standards (5–200 pg per well), a quality control extract, the test extracts and tracer (Hydrocortisone [Cortisol, (1,2,6,7-3H [N])-], Perkin-Elmer Life Sciences, Boston, MA, USA) were added in duplicate, and the plate was incubated overnight at 4 °C. Bound hormone was separated from free hormone by decanting the extract and washing the wells in RIA buffer. After the addition of 200 μl scintillation cocktail, the plate was counted on a beta-counter (Top-Count, Perkin-Elmer Life Sciences, Boston, MA, USA). In total we run 3 plates.

2.3. Wool cortisol analyses by AlphaLISA method

The wool cortisol from lambs evaluated by the AlphaLISA immunoassay method was measured using a commercial kit from Perkin-Elmer Life Science (Product number: AL314 HV/C/F, Boston, MA, USA) that in regard to the manufacturer's instructions for wool was slightly modified. The concentrations of the following reagents provided by the kit have been modified as follows: 5X Anti-Cortisol Antibody, 0.025 nM final; 5X Acceptor beads, 10 μg/mL final; 5X MIX, SA-Donor Beads (10 μg/mL final) + Biotinylated Cortisol-Tracer (8.332 nM final). Briefly, standards (7–720 pg per well), controls and samples (20 μl/well) and 10 μl of 5X Anti-Cortisol Antibody were pipetted into wells of a half-area 96-well microplate (Perkin-Elmer Life Science, Boston, MA, USA) in duplicate. After an incubation of 60 min at 23 °C, 10 μl of 5X Acceptor beads were added. Following a subsequent incubation step of 120 min at 23 °C, 10 μl of 5X MIX, SA-Donor Beads + Biotinylated Cortisol-Tracer were added. The microplate was read using Ensight Multimode plate reader (Perkin-Elmer Life Science, Boston, MA, USA) after a four-hour incubation in the dark. In total we run 3 microplates.

2.4. RIA and AlphaLISA validation tests

All the validation tests used different pool of samples constituted by five wool extracts and they were analysed by quintuple.

The parallelism test that consisted of determining the deviation from the standard curve of a series of wool extracts containing known amounts of cortisol, were prepared by serial dilution of wool extracts from animals that showed high concentrations of cortisol. Linear regression was used to determine if wool extracts and the standard cortisol curve deviated from parallelism.

The recovery test was conducted to evaluate the system response to an increasing amount of cortisol standard added to a wool extract with low cortisol. The percentage of recovery was determined as follows: [(measured cortisol in spiked sample)/(measured cortisol in non-spiked samp1e + cortisol added) x 100].

The sensitivity of the curve was calculated as the hormone concentration resulting in a displacement of the labelled hormone at least 2 standard deviations from maximal binding.

Precision was estimated by repeatedly assaying samples in the inter- and intra-assay and was expressed as the coefficients of variation (CV%).

2.5. Statistical methods

Statistical analyses were performed using R software, vers. 3.4.0 (R Core team, 2017), and the packages: DescTools (Signorell et al., 2017), mcr (Manuilova et al., 2014), BlandAltmanLeh (Lehnert, 2015) and lmtest (Zeileis and Horthorn, 2002) were considered. The normality of data distribution was tested using Lilliefors corrected Kolmogorov-Smirnov test. Since the differences between pairs (individual sample assessed with both RIA and AlphaLISA) were normally distributed, a paired t-test was used to assess the differences of wool cortisol concentration obtained by RIA and AlphaLISA between the overall means, and between the means within age of animals. The variables, RIA and AlphaLISA, do not meet the assumptions of independent samples t-test, hence, the effect of animals age (A0 vs. A2) on wool cortisol concentration was assessed using Mann–Whitney U test. The agreement of the wool cortisol concentration measured by RIA and AlphaLISA was assessed using Kendall's concordance coefficient W, from which the average Spearman correlation coefficient was obtained as reported in Howell (2002). Moreover, in order to assess this agreement, Bland-Altman plot (Giavarina, 2015) and the Passing-Bablok regression analyses were performed. The linearity of residuals was tested by Harvey-Collier test.

3. Results

3.1. RIA and AlphaLISA validation

The parallelisms between the wool dilution curves and the standard curves indicated that wool cortisol and standard cortisol reacted identically with the antibodies because high correlations ($r = 0.99$) were observed between the concentrations obtained and those expected both by RIA (Figure 1) and AlphaLISA (Figure 2). The relationships between wool cortisol concentrations and the standard cortisol curves were given by the equation y = 1.014x-3.133 for RIA assay and by the equation y = 1.009x-0.064 for AlphaLISA assay.

The recovery tests used to evaluate the response of the systems to an increasing mass weight of cortisol standards revealed for RIA and

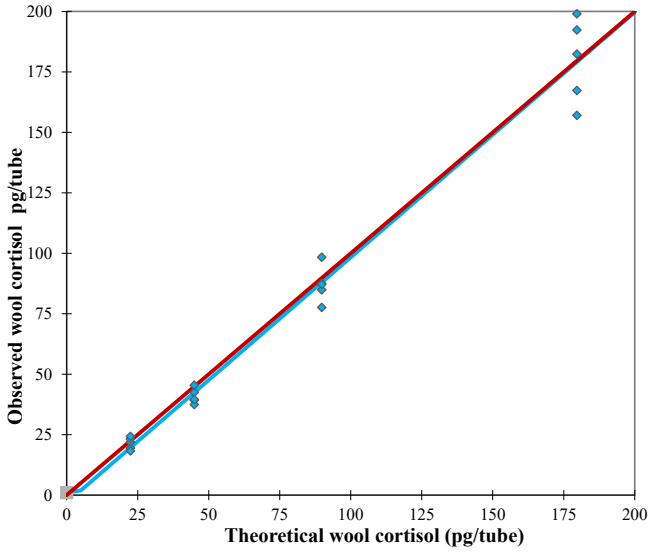

Figure 1. Graphical plot for the parallelism obtained by RIA. The relationship between wool cortisol concentrations and the standard cortisol curves was given by the equation y = 1.014x-3.133.

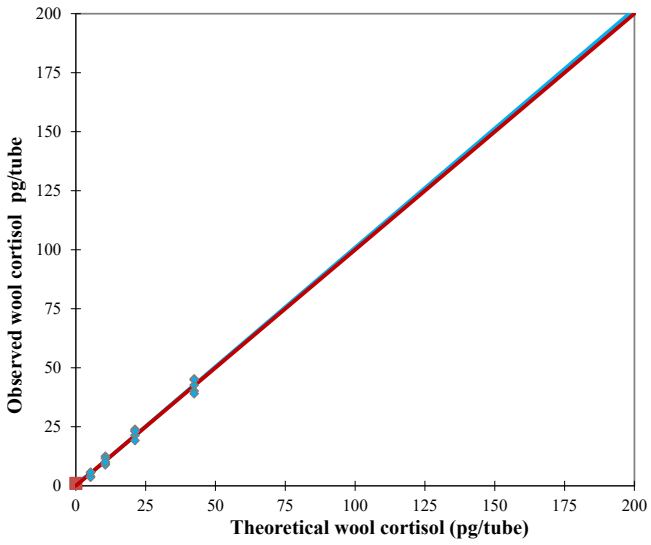

Figure 2. Graphical plot for the parallelism obtained by AlphaLISA assay. The relationship between wool cortisol concentrations and the standard cortisol curves was given by the equation y = 1.009x-0.064.

AlphaLISA recovery rates of 95.9 ± 8.8% (mean ± SD) and 94.6 ± 7.2% (mean ± SD), respectively.

The assay sensitivities were 24.6 and 17.0 pg/ml for RIA and AlphaLISA, respectively. A wool extract, in repeated determinations, showed intra- and inter-assay coefficients of variation of 4.6 and 8.7% and 3.2 and 11.3% for RIA and AlphaLISA assays, respectively.

3.2. RIA vs. AlphaLISA wool cortisol concentrations

The wool cortisol concentrations assessed with RIA were significantly higher than those assessed with AlphaLISA ($P < 0.01$; Table 1). The same result was obtained comparing the two methods within animals age, A0 (mean ± SE) (12.52 ± 1.92 vs. 9.40 ± 1.87 pg/mg for RIA and AlphaLISA respectively; $P < 0.01$) and A2 (6.36 ± 0.36 vs. 3.67 ± 0.30 pg/mg for RIA and AlphaLISA respectively; $P < 0.01$; data not reported in Tables). However, both methods were able to detect significant differences between wool cortisol concentrations in A0 and in A2 ($P < 0.01$; Table 2).

Considering agreement and association between RIA and AlphaLISA, Kendall's $W = 0.879$ ($P < 0.01$) and $r = 0.878$ (average Spearman correlation coefficient), suggesting that the two variables are strongly correlated (Schober et al., 2018). Using Passing Bablock regression analyses, the intercept was -2.04 (confidence interval 95%, CI, from -2.88 to -1.47) and the slope was 0.93 (CI, from 0.82 to 1.02; data not reported in Tables). Since the intercept was different from 0 and the slope was not different from 1, a constant, but not a proportional error was shown (Bilić-Zulle, 2011). In order to describe the agreement between RIA and AlphaLISA in more detail, Bland-Altman plot was considered. In this plot the previously highlighted constant error is evident (Figure 3). The mean difference between wool cortisol concentration assessed with RIA and AlphaLISA was 2.86 pg/mg (CI, from 2.48 to 3.23 pg/mg). Moreover, Bland-Altman plot showed a CI from -0.65 to 6.36 pg/mg that included all (89/89) the differences between RIA and AlphaLISA (Figure 3).

4. Discussion

In this study, we adapt and compare the Perkin-Elmer's cortisol AlphaLISA kit with a competitive solid-phase cortisol RIA assay already validated by our team in other species for measurement of lamb's wool cortisol concentration. Both the RIA and the AlphaLISA assays showed good precision, sensitivity and specificity for determining cortisol concentrations.

Table 1. Wool cortisol concentrations of lambs (*Ovis aries*; n = 89) obtained using Radio Immune Assay (RIA) and Amplified Luminescent Proximity Homogenous Assay-Linked Immunosorbent Assay (AlphaLISA).

Method	Wool cortisol concentration (pg/mg)					
	Mean	SD	CI, 95%	Median	Minimum	Maximum
RIA	8.78	7.94	7.11–10.46	6.57	2.37	58.02
AlphaLISA	5.93	7.61	4.32–7.53	3.77	0.96	52.15

SD: standard deviation; CI: confidence interval.

Table 2. Median [min, max] of wool cortisol concentration assessed with Radio Immune Assay (RIA) and Amplified Luminescent Proximity Homogenous Assay-Linked Immunosorbent Assay (AlphaLISA) as affected by lamb (*Ovis aries*) age.

	Age		*P*-value
	A0	A2	
Cortisol, pg/mg RIA	8.01 [4.43; 58.02]	5.98 [2.37; 13.53]	<0.01
Cortisol, pg/mg AlphaLISA	5.44 [1.53; 52.15]	3.22 [0.96; 11.06]	<0.01

A0: wool samples collected at birth from 35 lambs; A2 wool samples collected at two months of age from 54 lambs.

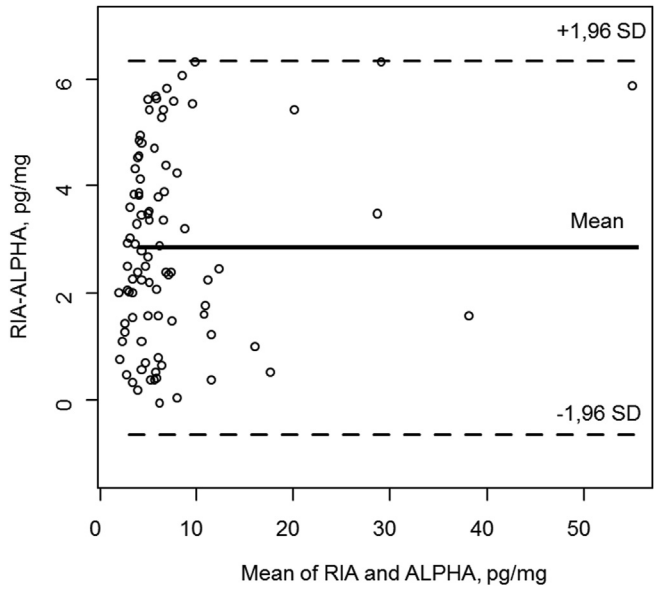

Figure 3. Bland-Altman plot that represents the comparison between Radio Immune Assay (RIA) and AlphaLISA for wool cortisol. Solid line indicates the mean of differences between methods; dashed lines indicate limits of agreement (mean ±1.96 standard deviation).

The modified cortisol AlphaLISA kit showed, as the RIA method, a high sensitivity that is necessary to measure hair or wool cortisol concentrations. Its high sensitivity is mostly due to the nature of the AlphaLISA detection platform: the flow of singlet oxygen produced upon donor beads irradiation induces remarkable signal amplification in nearby acceptor beads. Moreover, the high antibody density on beads creates an avidity phenomenon, increasing sensitivity (Beaudet et al., 2008). Along with the miniaturization of the assay, as it requires small sample volumes, AlphaLISA method decreases also the total assay time if compared with other analytical immunoassays. This advantage could be exploited in fast screenings of the farms.

Comparing the AlphaLISA with the RIA assay we found a strong correlation ($r = 0.878$) between cortisol concentrations measured using the two analytical methods, and the regression shows a constant and not proportional error was present.

The wool cortisol concentrations obtained by RIA were greater than the AlphaLISA's. This could be due to the diversity in the dosage steps but also to the diversity of the various molecules used in the two analytical methods: among these the use of different antibodies with different specificity and therefore different cross-reactivity towards unknown immunoreactive compounds (Beaumier et al., 1986). The mean difference between wool cortisol concentration assessed with RIA and AlphaLISA was 2.86 pg/mg but it is generally known that the absolute hair or wool cortisol values amongst studies of the same species that use different immunoassays should be always referred to the method and the antibody used. Certainly, both RIA and AlphaLISA were able to detect significant differences between the high and the low wool cortisol concentrations assessed in lambs at birth and at two months of age. However, since this is a study concerning the development of an analytical method, we think that a specific study is needed to investigate wool cortisol concentrations in lambs.

The purpose of the description and validation of both the techniques was to make them both available considering the different lab equipment and funds available in the worldwide scientific community.

5. Conclusions

The above-presented data support the validity of using AlphaLISA as an alternative method to RIA for the quantification of cortisol in sheep wool, a sample that requests high sensitivity of the analytical method and the capability to measure low hormone concentrations. AlphaLISA showed to be a technique that allows the detection of the molecule of interest in a highly sensitive, quantitative, reproducible and user-friendly mode with the possibility to be automised too. It does not request washing steps allowing thus to obtain robust performances. Considering the growing interest of a non-invasively collected and retrospective sample in domestic and wild animals as well primates, the AlphaLISA technology has a great potential to be further applied as an excellent tool to evaluate HCC in samples derived from animal species.

Declarations

Author contribution statement

T. Peric, A. Comin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Corazzin: Analyzed and interpreted the data; Wrote the paper.

M. Montillo: Performed the experiments.

A. Prandi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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