BMC Veterinary Research

RESEARCH

Open Access

Cortisone in saliva of pigs: validation of a new assay and changes after thermal stress

María Botía[1], Eva Llamas-Amor[1], José Joaquín Cerón[1*], Guillermo Ramis-Vidal[2], Andreu L. López-Juan[3], Juan L. Benedé[3], Damián Escribano[1,2], Silvia Martínez-Subiela[1] and Marina López-Arjona[4]

Abstract

Background Cortisone is derived from cortisol through the action of the enzyme 11β-hydroxysteroid dehydrogenase type II, and it has gained importance in recent years as a biomarker of stress. This study aimed to develop and validate an assay for the measurement of cortisone in pig saliva and evaluate whether its concentration varies in stressful situations. For this purpose, a specific immunoassay was developed and validated analytically, and a study was performed to evaluate whether cortisone concentrations in saliva can vary under heat stress conditions.

Results The assay proved to be accurate, reliable, and sensitive for the measurement of cortisone in pig saliva. The limit of detection of the assay was set at 0.006 ng/ml, and the lower limit of quantification was 0.023 ng/ml. It also correlated significantly with the results obtained by LC–MS/MS ($P = 0.003$; $r = 0.64$). In addition, the cortisone concentration in animals subjected to prolonged heat stress decreased significantly 15 days after treatment ($P < 0.0001$).

Conclusions According to these results, cortisone measured by this assay could be used as a tool for the non-invasive evaluation of thermal stress in pig saliva.

Keywords Cortisone, Biomarker, Heat stress, Saliva, Porcine

Background

Pigs are exposed during their productive system to many situations that can produce stress, leading to negative effects on animal health and production [1]. One of the most significant challenges facing the pig industry in recent years is heat stress caused by rising temperatures due to climate change. This phenomenon is responsible for significant economic losses within the sector [2].

Saliva biomarkers can be a suitable tool for the evaluation and control of stress, having the advantage of an easy collection that is not painful to the animals. Cortisol is the most commonly used biomarker in saliva to evaluate stress. However, the measurements of other biomarkers can provide additional information about the stress condition [3, 4].

One of these additional biomarkers is the cortisone, which is derived from cortisol by the action of the enzyme 11β-hydroxysteroid dehydrogenase type II (11β-HSD type II) [5]. Cortisone in addition to be described to increase in stressful situations specially in humans [6, 7], can be used for the calculation of the cortisone/cortisol ratio (Cn/C ratio) for the estimation of 11β-HSD type II enzyme activity.

*Correspondence:
José Joaquín Cerón
jjceron@um.es
[1] Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, Regional Campus of International Excellence Campus Mare Nostrum, University of Murcia, Murcia 30100, Spain
[2] Department of Animal Production, Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, Campus de Espinardo S/N, Murcia 30100, Spain
[3] GICAPC Research Group, Department of Analytical Chemistry, University of Valencia, Valencia, Burjassot 46100, Spain
[4] Department of Animal and Food Science, Universitat Autònoma de Barcelona, Barcelona, Cerdanyola del Vallès 08193, Spain

Previous research has demonstrated a pronounced elevation in the activity of the enzyme 11β-HSD type II in hair of pigs under conditions of high environmental temperature, accompanied by a notable increase in hair cortisone levels. While cortisol values remained unaltered by the elevated ambient temperature [8, 9]. This indicates that cortisone is a more sensitive biomarker of heat stress than cortisol in pig hair. However, hair is normally used for the assessment of chronic or long-term stress, whereas saliva allows us to assess stress produced in shorter times. Moreover, processing hair samples for glucocorticoid extraction is much more laborious and time-consuming than processing saliva, which is ready for analysis by centrifugation.

The use of enzyme immunoassays (EIAs) for the determination of salivary cortisone has been described in human [10]. In addition, cortisone can be measured in saliva by liquid chromatography coupled to mass spectrometry (LC–MS/MS) [11–14]. In recent years, AlphaLISA technology (PerkinElmer, Inc., MA, USA), based on amplified luminescence by the proximity of donor and acceptor beads, has become an immunoasay increasingly used in biomarker quantification due to the use of low sample volumes and the no need of washings steps. However, to the best of our knowledge, no immunological assays have been developed and used for the measurement of cortisone in the saliva of the pig.

Thus, this study aimed to (1) develop and validate a method based on AlphaLISA technology for the measurement of cortisone in pig saliva and (2) perform a pilot study to evaluate whether cortisone concentrations in saliva can vary under heat stress conditions and compare them with changes in cortisol, which is considered the classical marker of stress. Additionally, the cortisone/cortisol ratio (Cn/C ratio) was calculated for the estimation of 11β-HSD type II enzyme activity.

Results

Analytical validation

The intra-assay precision demonstrated a CV of less than 10%, while the inter-assay precision exhibited a CV of less than 15% (Table 1). The sample tested was linear after serial dilutions, showing a coefficient of determination of R2 = 0.9819 (Fig. 1). Furthermore, the mean recovery test was 95,5% for the high concentration sample and 97.3% for the low concentration sample (Table 2). The LOD was set at 0.006 ng/ml, and the LLQ was set at 0.023 ng/ml. The cross-reactivity of the assay was 6.2% for cortisol.

Method comparison

The values obtained by each method for each animal included in the study are shown in Table 3. No significant differences were observed between the different time points, although there was a tendency for cortisone to increase after stress. Regression analyses demonstrated that cortisone levels measured by the AlphaLISA assay showed a significant positive correlation with those measured by LC–MS/MS ($P = 0.003$; $r = 0.64$).

Changes in thermal stress

Salivary cortisone concentrations measured with the AlphaLISA method showed a significant increase in pigs with HS (median: 1.30 ng/ml; 25-75th percentile: 0.87–1.91 ng/ml) ($P < 0.0001$) compared with 15 days post-treatment (HS+15) when the same pigs were not under thermal stress (median: 0.69 ng/ml; 25-75th percentile: 0.52–0.87 ng/ml). These results are shown in Fig. 2.

Salivary cortisol concentrations did not significantly differ between heat stress (median: 140.5 ng/ml; 25-75th percentile: 118.9–190.8 ng/ml) and 15 days after treatment (median: 151.2 ng/ml; 25-75th percentile: 133.4–200.1 ng/ml), when the patients fully recovered (Fig. 3).

Salivary Cn/C ratio showed a significant increase in pigs with HS (median: 6.1×10^{-3}; 25-75th percentile: 4.4×10^{-3}-9.3×10^{-3}) ($P < 0.001$) compared with 15 days post-treatment (HS+15), when the same pigs were not under thermal stress (median: 4.6×10^{-3}; 25-75th percentile: 3.6×10^{-3}-5.9×10^{-3}). These results are shown in Fig. 4.

Discussion

The development and validation of a new method based on AlphaLISA technology for the detection of cortisone in pig saliva was performed in the present study. The low LOD (0.006 ng/ml) and LLQ (0.023 ng/ml) obtained by the method are indicative of the high sensitivity of the assay allowing the detection of low levels of cortisone in saliva. These values are below those provided by the user manual of other commercial ELISA kits, where the

Table 1 Mean cortisone concentration, standard deviation (SD), and intra- and inter-assay coefficients of variation (CVs) for saliva samples with low and high concentrations of cortisone measured with AlphaLISA

Sample	Intra-assay			Inter-assay		
	Mean (ng/ml)	SD	CV (%)	Mean (ng/ml)	SD	CV (%)
Low cortisone	0.39	0.03	8.89	0.42	0.054	12.85
High cortisone	0.97	0.04	4.45	1.14	0.066	5.78

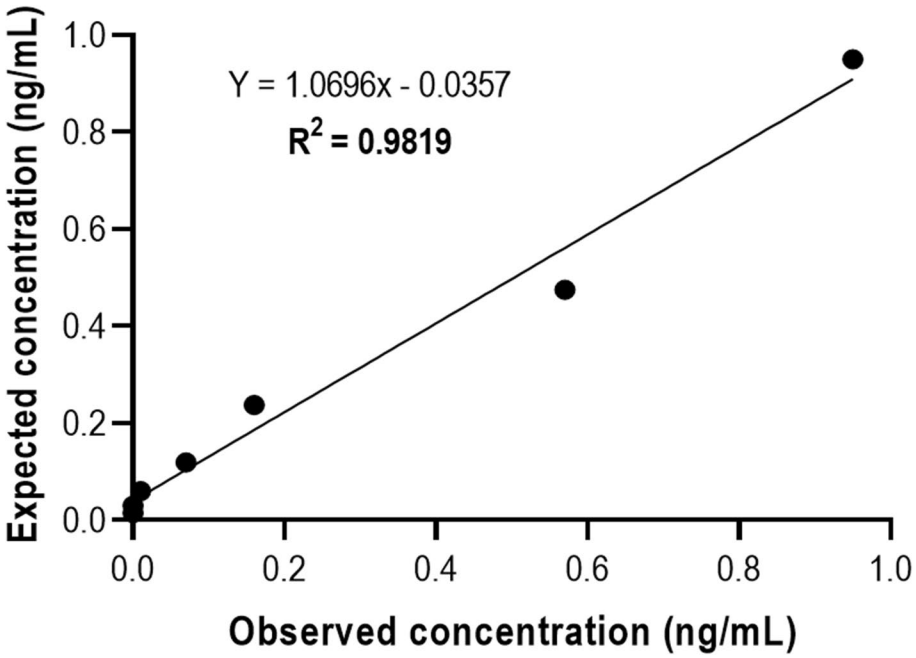

Fig. 1 Regression lines showing cortisone concentrations (ng/ml) in the AlphaLISA assay for a sample under dilution. The coefficient of determination (R^2) is shown in the figure

Table 2 Recovery of cortisone in saliva samples

	Sample		Expected (ng/ml)	Detected (ng/ml)	Recovery (%)
	Serial dilution	Constant dilution			
High concentration	1:2	1:4	0,97	0,89	91,81
	1:4	1:4	0,55	0,49	88,66
	1:8	1:4	0,37	0,40	106,14
Low concentration	1:2	1:4	0,34	0,34	98,86
	1:4	1:4	0,28	0,31	113,03
	1:8	1:4	0,25	0,20	80,01

Table 3 Individual results of salivary cortisone concentrations obtained by each measurement method. The results are expressed in ng/ml

	Animal	TB	T0	T15	T30
AlphaLISA	1	0.73	0.59	1.32	0.57
	2	0.63	0.47	0.41	0.96
	3	0.60	0.68	1.27	0.52
	4	0.64	0.56	0.70	0.50
	5	0.62	1.00	0.49	0.46
LC–MS/MS	1	0.85	0.61	1.10	0.48
	2	0.34	0.42	0.35	0.52
	3	0.21	0.27	0.83	0.44
	4	0.23	0.18	1.00	0.14
	5	0.19	0.34	0.27	0.28

LOD is as high as 0.93 ng/ml (cortisone ELISA kit, F9055, LSBio, Shirley, MA) and the LLQ is 0.78 ng/ml (cortisone ELISA kit, OKEH02619, Aviva Systems Biology, San Diego, CA). This data, together with the high correlation coefficients obtained in the linearity and recovery tests, make this assay valid for the measurement of cortisone in pig saliva samples with high specificity. Furthermore, the AlphaLISA technology offers a number of advantages over the enzyme-linked immunosorbent assay. These include a shorter incubation time and the elimination of the need for washing between steps. Additionally, a smaller sample volume (5 µl) is required [15].

In addition, the intra- and inter-assay CVs are both below the recommended 20% [16], similar to those previously obtained for other analytes in pig saliva by

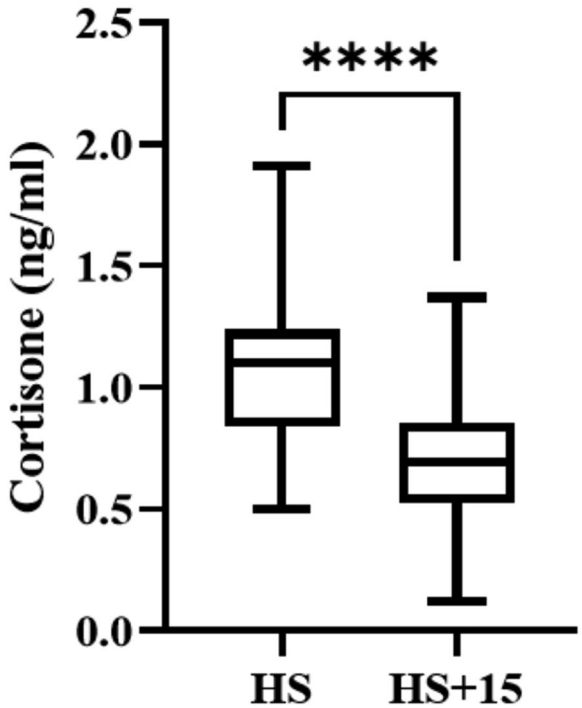

Fig. 2 Changes in cortisone concentrations at different times: at the time of heat stress (HS) and 15 days posttreatment (HS+15) according to the AlphaLISA method. Asterisks indicate significant differences (****$P < 0.0001$). The plots show the median (line within the box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers)

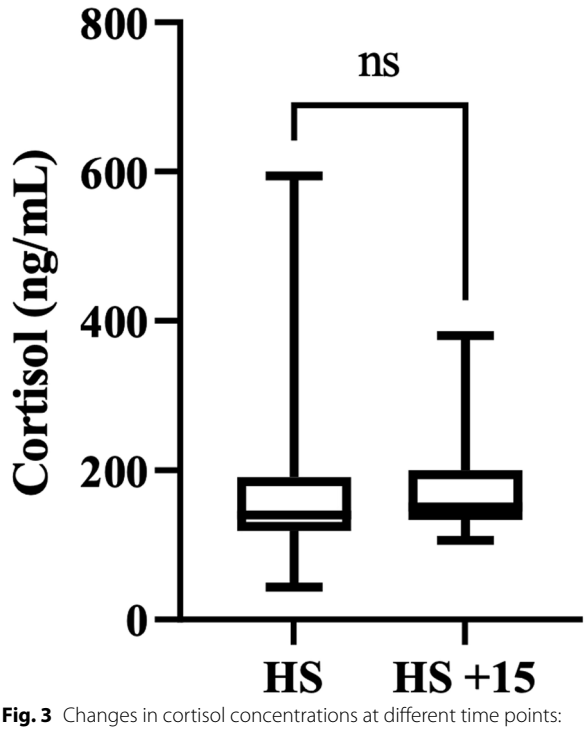

Fig. 3 Changes in cortisol concentrations at different time points: at the time of heat stress (HS) and 15 days after treatment (HS+15) according to the AlphaLISA method. "ns" indicates no significant differences. The plots show the median (line within the box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers)

AlphaLISA [15, 17]. Furthermore, the linearity of serial sample dilution indicated that the assay could detect cortisone accurately. On the other hand, this anti-cortisone antibody presents a 6.2% cross-reactivity with cortisol, which can be considered of low clinical significance [18].

The immunoassay of this study was compared to LC–MS/MS, which is considered the most specific method for measuring steroid hormones due to its high analyte specificity [19, 20]. In this comparison two interesting findings were obtained. One is that the correlation between assays was significant. However, this correlation was moderate. It could be postulated that this moderate correlation could indicate that the assay developed in this report could have some degree of cross-reactivity with other molecules. These molecules in some cases of other analytes such as the oxytocin can be related with metabolites or different forms of the same analyte [21], that are not detected by LC–MS/MS. This could be the reason why LC–MS/MS revealed a mean cortisone concentration of 0.45 ± 0.28 ng/ml, which is lower than that detected by the AlphaLISA (0.67 ± 0.26 ng/ml); being this fact in agreement with other studies where the immunoassays overestimated the LC–MS/MS values [22].

The other finding is that the nasal snare model included in this trial did not produce significant increases in cortisone at any time, probably because the peak increases in cortisone appeared in the pigs at different point times after the stimulus. However, the stress due to high temperatures produced significant increases in the animals. This could indicate that there could be variations in the cortisone response depending on the type of stress, and that cortisone could be more sensitive to detect stress due to high temperatures in comparison with other situations of stress such as nose snaring.

A significant increase in the salivary cortisone concentration in heat-stressed pigs were detected with the assay developed in this study. However, cortisol levels did not change significantly between before and after the heat shock. This would indicate that salivary cortisone is a sensitive biomarker of heat stress in pigs. It is of interest to point out that although in pigs the cortisone does not fulfil the premise that it is in higher values than cortisol (which has been described in humans as a reason for cortisone to be more sensitive to stress detection) [6]. It is more sensitive than cortisol to detect stress by high temperatures. These results are also in agreement with those observed in a previous study, in which cortisone, but not

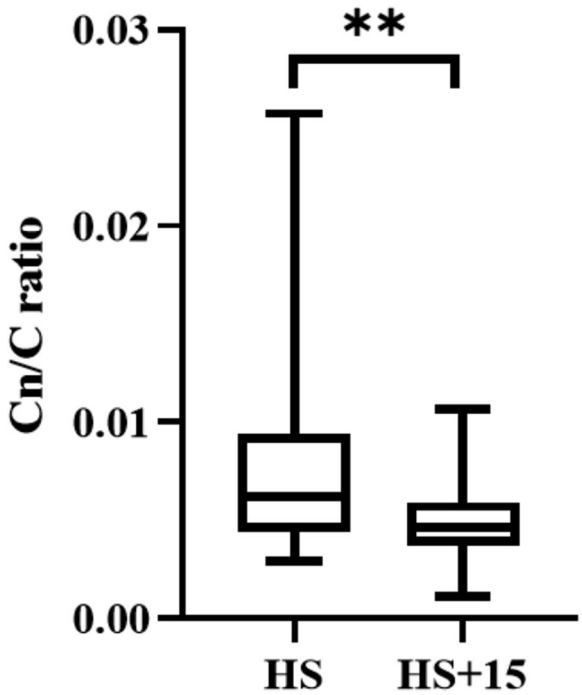

Fig. 4 Changes in Cn/C ratio at different time points: at the time of heat stress (HS) and 15 days after treatment (HS+15) according to the AlphaLISA method. Asterisks indicate significant differences (**$P < 0.001$) The plots show the median (line within the box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers)

cortisol, in pig hair was more abundant in heat-stressed pigs than in non-heat-stressed pigs [8]. The increase in cortisone and the lack of increase in cortisol could be due to the activation of the enzyme 11β-HSD type II. Although the reason why cortisol does not increase in saliva is not clear and further research should clarify this, some previous reports in hair have indicated that the concentration of cortisol in a situation of heat stress could reflect two factors: (1) its conversion to cortisone by the activation of the enzyme 11β-HSD type II which is increased and (2) the production of cortisol from cortisone by the enzyme 11β-HSD type I enzyme which is not inactivated in this situation. Overall, this leads to a situation in which cortisol production would continue, but to a lesser extent than cortisone production [8].

The Cn/C ratio was used to estimate the activity of the 11β-HSD type II enzyme. This ratio showed significantly higher results at the time of heat shock, which could indicate a higher enzymatic activity at that time. This will agree with a kinetic study of 11β-HSD in rat submandibular salivary gland which revealed that the enzyme 11β-HSD type II exhibited increased activity as temperature was elevated [23]. This increase reached its maximum also at 40 °C, a temperature similar to that experienced by the animals of our study. Since cortisone showed a higher magnitude of increase in pigs with heat stress, this would be preferable to Cn/C ratio to detect this condition.

This report has some various limitations. Further studies should clarify the reasons for not a higher correlation between the assay developed in this report and LC–MS. In addition, it would be of interest to evaluate how cortisone and cortisone/cortisol ratio behave in other situations of stress different to those of this manuscript.

Conclusion

Cortisone concentrations can be measured in pig saliva with the method developed in this study in an accurate and reliable way. Under our experimental conditions, this method was able to detect cortisone variations produced in heat stress situations where cortisol did not significantly change. Further studies should be carried out to gain knowledge about the possible applications of cortisone as a stress marker in pigs.

Materials and methods

Anti-cortisone antibody production

A sheep from the Veterinary Teaching Farm of the University of Murcia located in Guadalupe (Murcia, Spain) immunized with cortisone conjugated to bovine serum albumin (BSA) (Cloud-Clone) was used to produce the anti-cortisone polyclonal antibody used in this assay. This antigen (200 μg) was homogenized in complete Freund's adjuvant for the first injection (SC in a volume of 0.5 mL) and in incomplete Freund's adjuvant for the second and subsequent injections [24]. One week after each immunization, several blood samples were collected, and ELISA screening was performed to determine antibody production. The collected serum was concentrated by centrifugation at 3000 rpm for 5 min, filtered through a 0.22 μm syringe filter and passed through a protein G column using a chromatography system (ÄKTA Pure, Cytiva) to obtain the polyclonal anti-cortisone antibody as described in other studies [17]. This procedure was approved by the Ethical Committee on Animal Experimentation of the University of Murcia (Approval number: CEEA-696/2021; Approval date: 12 March 2021).

Cortisone AlphaLISA assay

AlphaLISA (PerkinElmer Inc., MA, USA) is a no-wash luminescent technology based on the proximity of two types of beads, acceptor and donor. For the measurement of cortisone in pig saliva, an indirect competitive AlphaLISA assay was developed with the polyclonal sheep anti-cortisone antibody described above. For optimization of the method, various concentrations of biotinylated cortisone-OVA conjugate at 0.5 mg/ml (Cloud-Clone) (1, 3, 6 and 9 nM), polyclonal anti-cortisone antibody at

17 mg/ml (5, 10 and 20 nM) and protein G acceptor beads at 5 mg/ml (10 and 20 µg/ml) were tested. The concentration of the donor beads was established according to the manufacturer's recommendations (20 µg/ml). A pig saliva sample of known concentration of cortisone (1.10 ng/ml) measured with LC/MS–MS [25] was used as a standard, and the calibration curve was generated with 8 standards at concentrations of 550, 275, 137.5, 68.75, 34.4, 17.2, 8.6, and 0 pg/ml. All dilutions of the reagents included in the assay were made with Alpha Universal buffer (PerkinElmer Inc., MA, USA). The results are expressed in ng/ml. The optimal conditions of the assay for cortisone measurement in pig saliva are shown in Fig. 5.

Assay validation

For the analytical validation of the method, the precision, accuracy and sensitivity were evaluated. In addition, cross-reactivity with cortisol and correlations with other methods were evaluated.

Analytical validation

Five samples were used for analytical validation. The concentration of each sample was determined using the assay developed in this study.

The precision was determined by the intra- and inter-assay coefficient of variation (CV). Two samples of different concentrations (0.39 and 0.97 ng/ml) were measured five times in the same assay for intra-assay precision, and another two samples (0.42 and 1.14 ng/ml) were

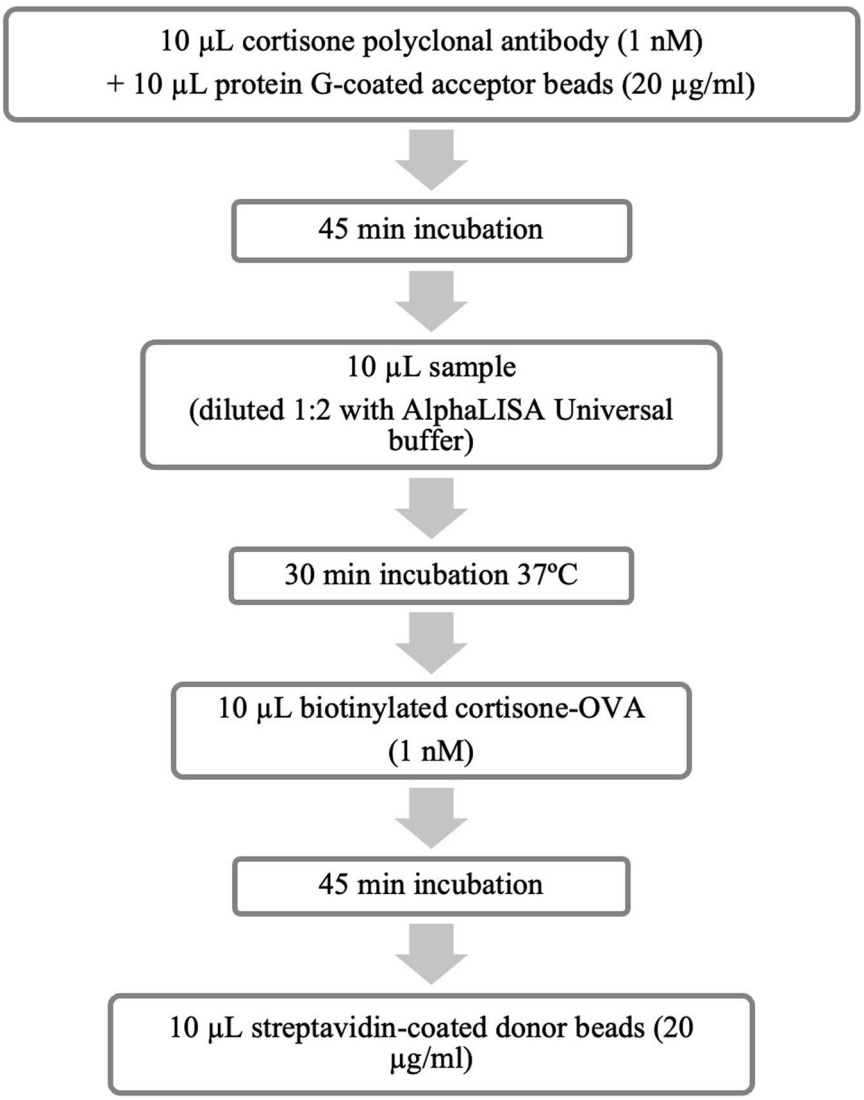

Fig. 5 AlphaLISA protocol for saliva cortisone measurement in pigs

measured five times on five different days using freshly prepared calibration curves for inter-assay precision [26]. The accuracy of the method was evaluated by lineaity of a sample of known concentration (0.95 ng/ml) diluted serially from 1:2 to 1:128 with the assay buffer. A recovery test was also made by adding different dilutions (from 1:2 to 1:8) of a pig saliva sample of known concentration of cortisone (0.45 ng/ml) to two samples with high (1,4 ng/ml) and low (0.23 ng/ml) concentration with constant dilution (1:4). Sensitivity was determined by the limit of detection (LOD), which was calculated as the mean plus two standard deviations of 15 replicate measurements of the assay buffer as described in other studies [15]. The lower limit of quantification (LLQ) was also determined as the lowest cortisone concentration that did not exceed 20% CV [9].

Cross-reactivity

For cross-reactivity calculations, 1000 ng of cortisol (cortisol solution, C-106, Merck) and cortisone (cortisone solution, C-130, Merck) diluted in methanol were serially diluted, and the signals and concentrations corresponding to each dilution were obtained by the assay developed in this study. Then, the cross-reactivity of the antibody with cortisol was calculated by the mean inhibitory concentration (IC50), understood as the ratio between the concentrations causing a 50% decrease in the detected signal by the method, according to the equation $\frac{Concentration\ of\ analyte\ giving\ 50\%\ B/B0}{Concentration\ of\ cross-reactant\ giving\ 50\%\ B/B0}$, where B is the signal given by the analyte or cross-reactant and B0 is the signal obtained in the absence of analyte [27, 28].

Method comparison

Five pigs housed at the Veterinary Teaching Farm of the University of Murcia located in Guadalupe (Murcia, Spain) were exposed to acute stress. Animals were subjected to restraint with a nasal snare for 1 min, and samples were taken before restraint (TB), during restraint (T0) and at 15 (T15) and 30 (T30) minutes after restraint. Cortisone was measured in a total of 20 samples in parallel by LC/MS–MS and the AlphaLISA technology-based assay developed in this study for comparison of results.

For LC–MS/MS analysis, miniaturized stir bar sorptive dispersive microextraction (mSBSDME), which was previously validated [29], was performed as a pretreatment for the samples. Then, an Agilent 1100 Series chromatography system coupled to an Agilent 6410B Triple Quad MS/MS was employed to measure cortisone in the mSBSDME extract. The separations were conducted on a Zorbax SB-C18 column (50 mm length, 2.1 mm i.d., 1.8 µm) from Agilent Technologies (Palo Alto, USA). Ten microliters of each solution were introduced into the chromatographic system. The mobile phase comprised solvent A (water, 0.5 mM ammonium fluoride) and solvent B (methanol) in a 50:50 (v/v) isocratic elution. The flow rate was maintained at 0.25 mL/min, and the column temperature was kept constant at 35 °C. The entire process was completed within a five-minute timeframe. The remaining conditions were as follows: gas temperature at 350 °C, nebulizer gas flow rate at 11 L min-1 and nebulizer gas pressure at 50 psi. Further information on the protocol used is available in the same study [29].

Changes in thermal stress

A total of 34 Large White pigs, aged 56 (±2) days, were exposed to high temperatures for one week due to a heat wave during the summer. All animals were housed in the transition unit of the Veterinary Teaching Farm of the University of Murcia, Guadalupe (Murcia), in pens with a minimum space allowance of 0.15 m^2/animal with water and feed available ad libitum. The average external temperature during the week of heat wave was 45 °C, and the internal temperature in the transition room reached 39 °C. Pigs included in the study showed symptoms related to heat stress, such as hyperthermia or fatigue, and inappropriate weight gain was observed (< 1 kg of weight gain in one week). Saliva samples were first collected when the pigs experienced symptoms of heat stress (HS); then, all pigs were treated with ovalbumin, and saliva was collected again 15 days post-treatment (HS+15), when they fully recovered.

In addition to cortisone, which was measured with the method developed in the study, cortisol concentrations were measured in all saliva samples for comparative purposes. Cortisol was measured with a method based on AlphaLISA technology previously developed and validated for use in pig saliva samples [30]. Furthermore, activity of 11β-hydroxysteroid dehydrogenase (11β-HSD) type II was estimated by cortisone/cortisol ratio as previously described [31].

Data analysis

The means, medians, and intra- and inter-assay CVs were calculated via routine descriptive statistical procedures and computer software (Microsoft Excel 2016). Linearity under dilution was investigated by linear regression. Pearson correlation coefficients were obtained for both assays using Graph Pad software (GraphPad Prism, version 9 for Windows, Graph Pad Software Inc., San Diego, USA). The statistical analysis of the clinical validation was also performed using GraphPad software. The Shapiro–Wilk test was performed to assess the distribution of the data, and the data followed a normal distribution. Thus, to compare the cortisone and cortisol levels before and after stressful situations, the Wilcoxon matched-pairs

signed-rank test was employed. The results are reported as medians and 25th–75th percentiles (in the text) and as line-box plots (in the figures). Values of $P < 0.05$ were considered significant.

Abbreviations

11β-HSD	11β-Hydroxysteroid dehydrogenase
LC–MS/MS	Liquid chromatography coupled to mass spectrometry
BSA	Bovine serum albumin
OVA	Ovalbumin
RT	Room temperature
CV	Coefficient of variation
LOD	Limit of detection
LLQ	Low limit of quantification
mSBSDME	Miniaturized stir bar sorptive dispersive microextraction

Authors' contributions

MB, SMS and MLA designed the study and contributed to the implementation of the research. MB and MLA developed and validated the cortisone assay. JLB and ALLJ performed the LC–MS/MS assay. ELA and GRV performed the heat stress model experiment. MB, MLA and DE performed the acute stress model experiment. MB, MLA and DE analysed the data, drafted the manuscript and designed the graphics. JJC and SMS were major contributors to the analysis of the results and to the writing and revision of the manuscript. All the authors have read and approved the final manuscript.

Funding

This research was funded by the European Union's Horizon 2020 Research and Innovation Program under grant agreement No. 862919. In addition it was funded by the Spanish "Agencia Estatal de Investigación" (Project PDC2021-121291-I00 funded by MCIN/AEI/10.13039/501100011033 and European Union Next GenerationEU/PRTR). María Botía (21789/FPI/22) and Eva Llamas Amor (22296/FPI/23) were funded by Fundación Séneca, Región de Murcia (Spain). Damián Escribano received financial support from the Ramón y Cajal program (RYC2022-037873-I) funded by the Spanish Ministry of Science and Innovation. ML-A has a postdoctoral fellowship, "Juan de la Cierva Formación", supported by the Spanish Ministry of Science and Innovation (FJC2021-047105-I), Spain.

Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval consent to participate

The Ethical Committee on Animal Experimentation (CEEA) of the University of Murcia approved the research protocol in this study with approval number CEEA-696/2021, according to the European Council Directives regarding the protection of animals used for experimental purposes. Also, this study complies with ARRIVE guidelines for the care and use of animals. Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 28 February 2024 Accepted: 16 July 2024
Published online: 19 August 2024

References

1. Contreras-Aguilar MD, Escribano D, Quiles A, López-Arjona M, Cerón JJ, Martínez-Subiela S, et al. Evaluation of new biomarkers of stress in saliva of sheep. Animal. 2019;13:1278–86.
2. St-Pierre NR, Cobanov B, Schnitkey G. Economic Losses from Heat Stress by US Livestock Industries. J Dairy Sci. 2003;86(SUPPL. 1):E52–77.
3. Cerón JJ, Contreras-Aguilar MD, Escribano D, Martínez-Miró S, López-Martínez MJ, Ortín-Bustillo A, et al. Basics for the potential use of saliva to evaluate stress, inflammation, immune system, and redox homeostasis in pigs. BMC Vet Res. 2022;18:1–17.
4. Martínez-Miró S, Tecles F, Ramón M, Escribano D, Hernández F, Madrid J, et al. Causes, consequences and biomarkers of stress in swine: an update. BMC Vet Res. 2016;12.
5. de Guia RM. Stress, glucocorticoid signaling pathway, and metabolic disorders. Diabetes Metab Syndr Clin Res Rev. 2020;14:1273–80.
6. Bae YJ, Reinelt J, Netto J, Uhlig M, Willenberg A, Ceglarek U, et al. Salivary cortisone, as a biomarker for psychosocial stress, is associated with state anxiety and heart rate. Psychoneuroendocrinology. 2019;101:35–41.
7. La Marca-Ghaemmaghami P, La Marca R, Dainese SM, Haller M, Zimmermann R, Ehlert U. The association between perceived emotional support, maternal mood, salivary cortisol, salivary cortisone, and the ratio between the two compounds in response to acute stress in second trimester pregnant women. J Psychosom Res. 2013;75:314–20.
8. Escribano D, Contreras-Jodar A, López-Arjona M, Cerón JJ, Fàbrega E, Aymerich P, et al. Changes in cortisol and cortisone in hair of pigs reared under heat stress conditions. Front Vet Sci. 2023;10.
9. López-Arjona M, Tecles F, Mateo S V., Contreras-Aguilar MD, Martínez-Miró S, Cerón JJ, et al. Measurement of cortisol, cortisone and 11β-hydroxysteroid dehydrogenase type 2 activity in hair of sows during different phases of the reproductive cycle. Vet J. 2020:259–260.
10. Anderson T, Wideman L. The association between the cortisol and cortisone awakening responses. Psychoneuroendocrinology. 2023;152:106075.
11. Ceglarek U, Werner M, Kortz L, Körner A, Kiess W, Thiery J, et al. Preclinical challenges in steroid analysis of human samples. J Steroid Biochem Mol Biol. 2010;121:505–12.
12. Antonelli G, Ceccato F, Artusi C, Marinova M, Plebani M. Salivary cortisol and cortisone by LC–MS/MS: validation, reference intervals and diagnostic accuracy in Cushing's syndrome. Clin Chim Acta. 2015;451:247–51.
13. Vieira JGH, Nakamura OH, Carvalho VM. Determination of cortisol and cortisone in human saliva by a liquid chromatography-tandem mass spectrometry method. Arq Bras Endocrinol Metabol. 2014;58:844–50.
14. Giergiel M, Olejnik M, Jabłoński A, Posyniak A. The markers of stress in swine oral fluid. J Vet Res. 2021;65:487–95.
15. López-Martínez MJ, Escribano D, Martínez-Miró S, Ramis G, Manzanilla EG, Tecles F, et al. Measurement of procalcitonin in saliva of pigs: a pilot study. BMC Vet Res. 2022;18:1–9.
16. Brunzendorf J, Behrens R. How to type test the coefficient of variation of an indication. Radiat Prot Dosimetry. 2007;123:21–31.
17. López-Arjona M, Mateo S V., Manteca X, Escribano D, Cerón JJ, Martínez-Subiela S. Oxytocin in saliva of pigs: an assay for its measurement and changes after farrowing. Domest Anim Endocrinol. 2020;70.
18. Krasowski MD, Drees D, Morris CS, Maakestad J, Blau JL, Ekins S. Cross-reactivity of steroid hormone immunoassays: clinical significance and two-dimensional molecular similarity prediction. BMC Clin Pathol. 2014;14:33.
19. Fariha R, Jabrah M, Hill C, Spooner A, Deshpande P, Tripathi A. Simultaneous detection of salivary cortisol and cortisone using an automated high-throughput sample preparation method for LC-MS/MS. SLAS Technol. 2022;27:237–46.
20. Antonelli G, Ceccato F, Artusi C, Marinova M, Plebani M. Salivary cortisol and cortisone by LC-MS/MS: validation, reference intervals and diagnostic accuracy in Cushing's syndrome. 2015. https://doi.org/10.1016/j.cca.2015.10.004.
21. MacLean EL, Wilson SR, Martin WL, Davis JM, Nazarloo HP, Carter CS. Challenges for measuring oxytocin: The blind men and the elephant? Psychoneuroendocrinology. 2019;107:225–31.
22. Jewgenow K, Azevedo A, Albrecht M, Kirschbaum C, Dehnhard M. Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results. Conserv Physiol. 2020;8.
23. Ferguson MM, MacPhee GB. Kinetic study of 11beta-hydroxysteroid dehydrogenase in rat submandibular salivary gland. Arch Oral Biol. 1975;20:241–5.
24. Delahaut P. Immunisation – Choice of host, adjuvants and boosting schedules with emphasis on polyclonal antibody production. Methods. 2017;116:4–11.

25. Grau J, Benedé JL, Chisvert A, Salvador A. Modified magnetic-based solvent-assisted dispersive solid-phase extraction: application to the determination of cortisol and cortisone in human saliva. J Chromatogr A. 2021;1652: 462361.
26. Escribano D, Fuentes-Rubio M, Cerón JJ. Validation of an automated chemiluminescent immunoassay for salivary cortisol measurements in pigs. J Vet Diagn Invest. 2012;24:918–23.
27. Wild D. The Immunoassay Handbook. Theory and Applications of Ligand Binding, ELISA and Related Techniques. 2013.
28. Sotnikov DV, Zherdev AV, Zvereva EA, Eremin SA, Dzantiev BB. Changing cross-reactivity for different immunoassays using the same antibodies: Theoretical description and experimental confirmation. Appl Sci (Switzerland). 2021;11:6581.
29. Azorín C, Benedé JL, Chisvert A. New challenges in sample preparation: Miniaturized stir bar sorptive dispersive microextraction as a high-throughput and feasible approach for low-availability sample preparation. Anal Chim Acta. 2023;1238.
30. López-Arjona M, Escribano D, Mateo SV, Contreras-Aguilar MD, Rubio CP, Tecles F, et al. Changes in oxytocin concentrations in saliva of pigs after a transport and during lairage at slaughterhouse. Res Vet Sci. 2020;133:26–30.
31. Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, Keevil BG. Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography–tandem mass spectrometry: Application in basal and stimulated conditions. J Chromatogr B. 2009;877:3771–5.

Publisher's Note