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Relationship between plasma, saliva, urinary and faecal cortisol levels in pigs

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ABSTRACT

Blood collection for assessment of stress markers such as cortisol, involves restraining and induction of stress on animals. The present study examined the relationship between circulating cortisol levels with its levels in other biological sources (saliva, urine, faeces) to assess utility of non-invasive methods of sample collection for stress assessment in crossbred pigs (Hampshire × Ghungroo). Urine samples were collected after 1 and 2 h of blood and saliva collection, whereas faecal samples were collected after 24 and 48 h of initial sample collection. Mean cortisol levels in plasma and saliva was positively correlated. The correlation between plasma cortisol and second hour mean urinary cortisol values was higher compared to first hour samples. The faecal reactive metabolite levels were weakly correlated to plasma, saliva and urinary cortisol levels. It is concluded that the salivary cortisol values reflect its plasma levels at the time of collection most closely amongst the biological samples studied.

Keywords: Cortisol, Faeces, Pig, Saliva, Serum, Urine

Pigs are subjected to various physiological, environmental and social stresses from birth to slaughter, resulting in decreased production performance such as average body weight gain, reduced feed intake (White *et al.* 2008), reproductive failure (Einarsson *et al.* 2008) and poor meat quality (Lebret *et al.* 2015), leading to major economic losses in pig farms. Several studies have reported estimation of cortisol levels in pigs for quantification of stress in blood (Marco-Ramell *et al.* 2011, Marco-Ramell *et al.* 2016, Carreras *et al.* 2017), saliva (Parrott and Misson 1989, Hay and Mormede 1998), urine (Hay and Mormede 1998, Pol *et al.* 2002), faeces (Mostl *et al.* 1999, Mostl and Palme 2002, Carlsson *et al.* 2007, Boon *et al.* 2015) and hair (Carroll *et al.* 2018). As the blood collection involves restraining and induction of stress on animals, the utility of estimation of cortisol through non-invasive methods with an added advantage of multiple sampling have been explored. One of the essential prerequisites of non-invasive methods is to establish the relationship between cortisol levels in the blood and other biological sources. Most of the previous studies have studied the relationship between corticosteroids between two biological sources, not multiple sources concurrently. Hence, the present study was conducted with an objective of examining the relationship between circulating cortisol levels with its levels in other biological sources (saliva, urine, faeces) obtained through non-invasive methods for stress assessment. A simple device developed for collection of saliva from animals is also described in the present paper.

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MATERIALS AND METHODS

Twenty crossbred pigs (Hampshire × Ghungroo), aged 12–18 months were reared at the institutional farm of the Indian Council of Agricultural Research-National Research Centre on Pig, Guwahati, Assam. The studies were conducted as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and was approved by institutional animal ethics committee. All animals were apparently healthy, maintained under standard feeding and management practices. Saliva samples were collected using a custom made bud device immediately prior to the blood collection. Pigs were restrained, salivary bud was inserted into the mouth, and concurrently blood was also collected from anterior venacava in heparinized tubes. Plasma separated from blood was stored at –80°C until cortisol estimations. Urine samples were collected at the end of one and two hours of blood collection, whereas the faecal samples were collected after 24 and 48 h of blood collection to assess cortisol levels. Pooled samples from individual animal were taken for the analysis. The sample collections were based on cortisol metabolism and excretion rates reported earlier (Mostl *et al.* 1999, Walker and Seckl 2001, Mostl and Palme 2002, Jung *et al.* 2014). All samples were collected between 10–11h to reduce influence the circadian fluctuations in the hormone levels and were repeated for two times in consecutive days.

Development of a simple device for collection and preservation of saliva: For easy collection of saliva, a simple device for collection of saliva was developed using cotton bud, a filtration tube and a microcentrifuge tube (Figs 1 and 2). The saliva was collected using the bud held in a

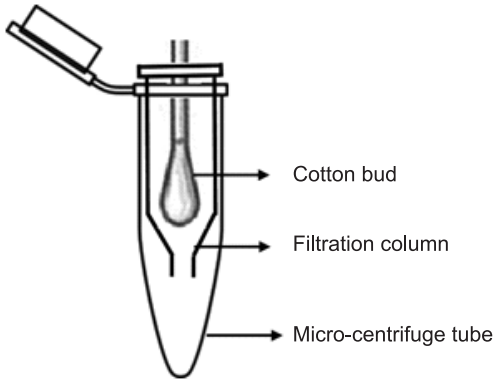


Fig 1. Line drawing of the saliva sample collection device.

forceps in the mouth of animal for 30 seconds, within which saliva was absorbed into the cotton bud. The bud was placed back in the microcentrifuge tube and transported to the lab. The bud containing saliva was placed into a new microcentrifuge tube, prefixed with a filtration column. The entire assembly was centrifuged at 4,000 rpm for 10 min to obtain clear saliva. The filtration tube and bud were disposed and saliva containing microcentrifuge was stored at –80°C for analysis.

Extraction of cortisol and ELISA: Cortisol was extracted from plasma samples by adding 2 ml diethyl ether to 100 µl of plasma as described earlier (Palme and Mostl 1997). The mixture was placed on a shaker for 60 minutes, supernatant was extracted and stored at –80°C for analysis. Cortisol concentrations in saliva was estimated directly except that the saliva was diluted 1: 10 in phosphate buffered saline (*p*H 7.4). The faecal samples were collected and processed for cortisol estimation as per method of Palme and Mostl (1997) with minor modifications. Briefly, faecal samples were dried at 60°C, crushed, and 0.5 g of the sample was suspended in 1 ml of 80% methanol. The mixture was vortexed, centrifuged for 15 min at 3,000 rpm and the supernatant collected was stored at –80°C until analysis. All cortisol estimations were done using a commercially available enzyme-linked immunosorbent assay kit (Labor Diagnostika Nord GmbH and Co. KG, Nordhorn, Germany). Urinary cortisol concentrations were expressed as a function of creatinine to correct for the difference in the dilution of urine excreted in relation to water consumption by the animal (Pol *et al.* 2002). Creatinine levels in urine was estimated by Jaffe`s reaction method (Toora and Rajagopal 2002).

Statistical analysis: The data was analysed using ANOVA followed by calculation of correlation coefficient between levels of cortisol in various biological samples. Pearson correlation coefficient and their significance were calculated using R environment (Version 3.5.1) in R studio (v 1.1.456) (Rstudio 2018; Rcore team 2018). The graphs were visualized using R package programme Corrplot V0.84 (Wei and Simko 2017).

RESULTS AND DISCUSSION

Among several biomarkers, cortisol level is the most commonly used indicators of stress in pigs (Martinez-Miro *et al.* 2016). Cortisol levels in plasma, saliva and faeces from pigs were estimated during two consecutive days to assess the suitability of various biological samples for estimation of cortisol that could be collected non-invasively (urine, saliva and faeces) to assess stress. Saliva was collected using developed device and the average yield of saliva was 424±16 µl per tube using a single bud. Two buds could be introduced into the same column to increase the yield of saliva proportionately. Alternatively, use of a larger bud also yielded increased amount of saliva. The correlation between holding capacity of bud and final yield of saliva through the procedure described was 0.93.

The results of cortisol levels and its correlation in different biological samples are shown in Fig. 3. The bivariate plot graphs indicates the relationship between cortisol levels in different biological samples measured on the individual samples, revealing the degree and pattern of relation between the two variables. Mean cortisol levels in plasma and saliva was 24.12±2.26 and 0.69±0.11 µg/dl, respectively. The ratio of plasma to salivary cortisol was 1: 0.03, which is comparable to previous reports (Bushong *et al.* 2000, Cook *et al.* 1996, Parrot *et al.* 1989). The correlation between circulating and salivary cortisol levels (r=0.77) was between values reported by Cook *et al.* (1996) (r=0.88) and Bushong *et al*. (2000) (r=0.60). The mean urinary cortisol values expressed as function of creatinine was 14.52±1.15 µg/g and was correlated to values in plasma (r=0.18) and saliva (r=0.17) respectively after one hour of blood collection. After the end of two hours, the urinary

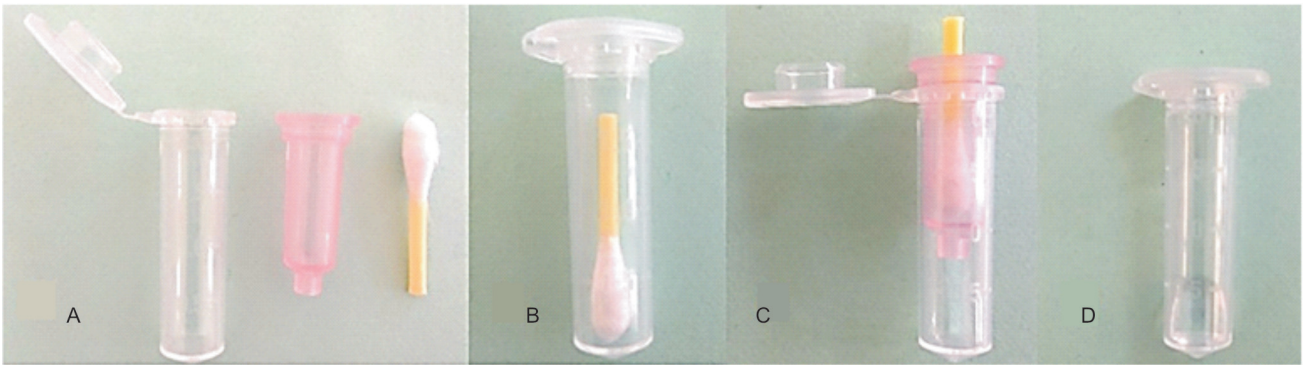


Fig 2. Saliva sample collection device. The saliva collected in the device (A) was transported (B) and centrifuged (C) and stored subsequently (D) until analysis.

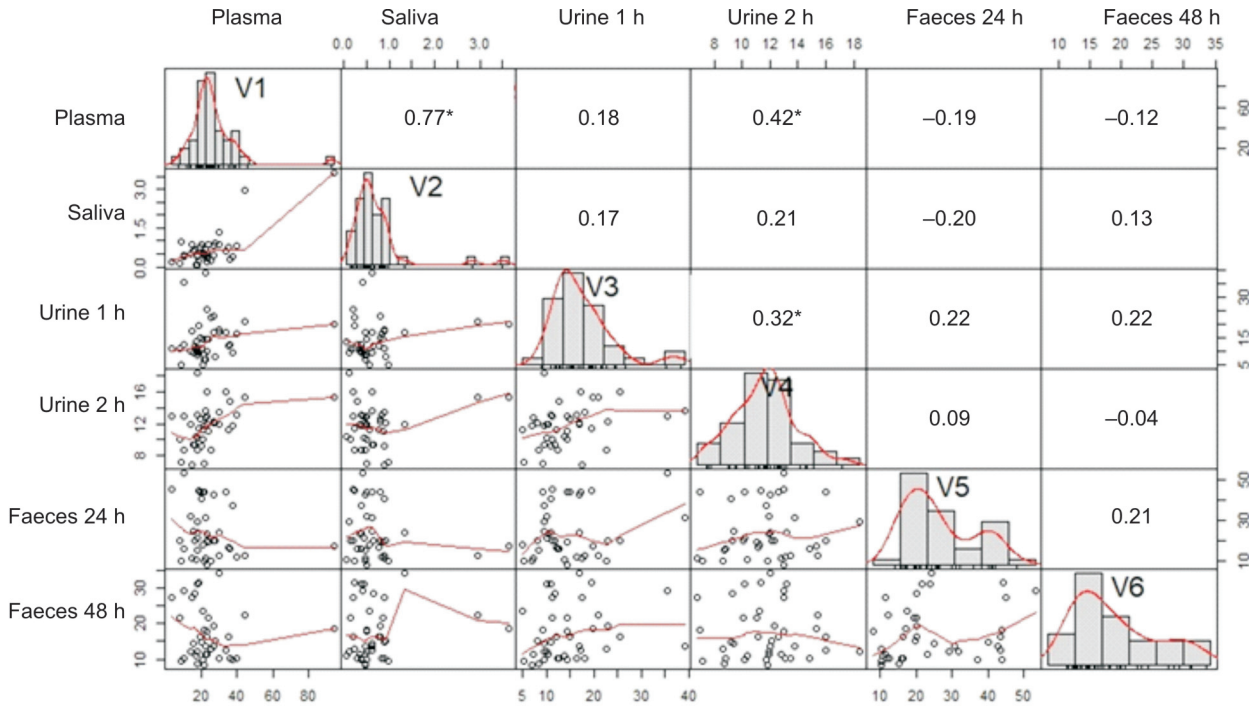


Fig. 3. Distribution of cortisol levels in pigs from biological sources. Distribution of hormone levels in different samples across the animals are shown as histogram. Bottom of the diagonal: the bivariate scatter plots showing relationship between two different samples with line fitting. Top of the diagonal: correlation values and significant ones are indicated with *(P<0.05). Values outside the frame shows the cortisol levels in difference. (Plasma and saliva-µg/dl; Urine- µg/g creatinine; Faeces- ng/g). The numbers V1 to V6 indicate source of cortisol as separate variables.

cortisol levels as expressed in terms of creatinine excretion decreased marginally (11.74±0.41 µg/g of creatinine), however, the correlation with plasma (r=0.42) and saliva (r=0.21), increased, similar to an earlier report (Jung *et al.* 2014), reflecting the metabolism of the hormone.

The overall faecal cortisol and other reactive metabolites values collectively at the end of 24 and 48 h was 24.36±2.02 and 17.32±1.20 ng/g, respectively. The faecal reactive metabolite levels after 24 h was weakly correlated to plasma, saliva and urinary (1 h) cortisol values (–0.19, –0.20 and –0.22 respectively). Similarly, the correlation between plasma, salivary and urinary (1h) with faecal cortisol and reactive metabolites was also insignificant (r = –0.12, 0.13 and 0.20, P>0.05), respectively. Results of the present study are in line with the observations made for cortisol estimations from different sources blood (Marco-Ramell *et al.* 2011, Marco-Ramell *et al.* 2016, Carreras *et al.* 2017), saliva (Parrott and Misson 1989, Hay and Mormede 1998), urine (Hay and Mormede 1998, Pol *et al.* 2002) and faeces (Mostl *et al.* 1999, Mostl and Palme 2002, Carlsson *et al.* 2007).

In the present study, the cortisol or/other reactive metabolites were estimated concurrently in plasma, saliva, urine and faeces in the same animals to obtain overall perspective of hormone levels in the body as compared to earlier studies (Parrott and Misson 1989, Hay and Mormede 1998, Mostl *et al.* 1999, Pol *et al.* 2002, Mostl and Palme 2002, Carlsson *et al.* 2007), where the estimations were restricted to few biological sources. Saliva sampled, almost at the same time as that of blood, urine and faeces collected at the end of 1 or 2 h and 24 or 48 h, respectively provides an opportunity to examine their utility for assessing short, mid and long term levels of cortisol/stress in pigs. Cortisol in saliva is in unbound active form, and is considered to be a good indicator of levels of blood cortisol (Hellhammer *et al.* 2009, Escribano *et al.* 2015).

The sampling intervals were chosen based on the metabolism of cortisol, approximately 93% of the circulating cortisol is eliminated in urine in pigs and rest through excretion via faeces in about 48 h. (Mostl and Palme 2002). Relatively higher correlation between plasma and salivary cortisol suggests that it can reflect the amount of free cortisol in the peripheral circulation. The increase in correlation between plasma and urinary cortisol levels at the end of 2 h as against 1 h samples (r = 0.18 vs 0.42) might also reflect the time for metabolism and excretion of cortisol in urine. On the other hand, low correlations between plasma, salivary and faecal cortisol levels suggest that the sampling at 1 h or 2 h and 24 h or 48 h, respectively could not sufficiently reflect levels in circulation as reported earlier (Palme and Mostl 1997, Mostl *et al.* 1999, Carlsson *et al.* 2007). In pigs, only 7% of the cortisol is excreted in faeces and a part of these metabolites are reported to be immunoreactive (Mostl *et al.* 1999). Besides this, a considerable variation in the excretion of cortisol in faeces after metabolism has also been reported (Palme *et al.* 1996). Hence, these could be reasons for the low correlation between plasma and salivary cortisol with its corresponding

faecal levels. The major factors that might have influenced faecal cortisol estimations in the present study is the presence of several metabolites resulting from body metabolism, their immunoreactivity and possible microbial transformations in the intestinal tract (Mostl and Palme 2002) and rate of passage of ingesta through the gastrointestinal tract (Cook 2012).

In general, the physiological status of the animals, handling stress at the time of sample collection, binding with proteins and reactivity of cortisol, other reactive substances in the biological samples to the hormone estimation method adopted in the study might have influenced the results. The variation in the correlation observed between plasma cortisol and other biological samples could have been, further modulated by actual levels of hormone, extent of metabolism and sample preparation methods. Hence, the correlations observed represent the cortisol values estimated in different biological sources collected at different time points and does not exclude the individual variations in the metabolism.

From the results of the study, it can be concluded that the salivary cortisol values reflect its plasma levels at the time of collection most closely amongst other biological samples studied. Urinary cortisol levels, almost 2 h after the blood collection were also partially suggestive of plasma cortisol levels. Salivary and urinary cortisol levels could be explored as a predictor of plasma cortisol levels, hence to indirectly assess acute and short term stress, respectively in pigs. The utility of faecal cortisol may require further studies for identification and quantification of metabolites along with time course of their excretion.

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