

CortiCow project: development of a rapid and non-invasive lateral flow immunoassay for the evaluation of cortisol levels in bovine saliva

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ABSTRACT

Being able to obtain an objective and repeatable measurement of stress levels from a single subject represents a primary issue in animal welfare science, especially in relation to cattle farming. A potential solution has been recently identified in the determination of cortisol's salivary levels: a non-invasive method strictly related to acute stress. The development of an on-field, easy-to-use method to perform the analysis is yet to be validated. In this study, we developed and tested the reliability of salivary cortisol as a marker for the evaluation of animal welfare. We aimed to develop an on-field use Lateral Flow Immunoassay (LFIA) for the evaluation of acute stress in bovines. The availability of this non-invasive diagnostic tool will facilitate monitoring of animal welfare. In the short term, this innovation is expected to assist farmers and veterinarians in performing a more objective evaluation of the animal's acute stress levels. In the long term, the device could become a key-instrument for the EU's growing necessity to better monitor and identify the potential stressor factors in animal farming.

Section: RESEARCH PAPER

Keywords: animal welfare; lateral flow immunoassay; cortisol; saliva; bovine; measurements

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1. INTRODUCTION

1.1. Animal welfare

The concept of animal welfare varied throughout the years: conceived as the fulfilment of the 5 freedoms [1], it has soon evolved into a wider concept aimed at providing “a life worth to be lived” to farmed animals and enriched with the “positive animal welfare” (WAP) philosophy [2]. The novelty of the WAP approach lies in evaluation of the animals' mental state, and not only of their physical state [2]. Animal welfare has received particular attention in livestock species due to an ethical obligation to maintain decent living conditions for production animals.

The topic currently presents a particular interest to the modern consumers. In Europe, the public's growing attention has led to a non-dismissible pressure on the main Institutions, ultimately causing several changes in the legislative frame of animal farming. The Council Directive EU 98/58 laid the

minimum standards (absence of unnecessary pain, suffering or injury) for the protection of animals bred or kept for farming purposes [3]. Since then, more actions have been subsequently taken in this direction, leading in particular to the regulations EU 1/2005 and EU 1099/2009, aimed at guaranteeing the respect of the animals' welfare during transportation and slaughtering [4], [5]. More recently, the EU Commission promoted a revision of the legislation on animal welfare with the purpose of establishing welfare indicators to monitor the animals' conditions over time [6]. Scientific investigations have been conducted in parallel to the legislative efforts, in order to identify an objective evaluation method to understand the animals' subjective physical and psychological responses to stressful external stimuli. The literature reports different approaches to the matter, ranging from on-farm evaluation of the animals' behavior to laboratory analysis of the main metabolic and hormonal patterns [7]-[9]. Currently, the scientific community is focusing on the identification of specific and non-invasive biomarkers able to

clarify the mechanisms by which the organism responds to stressors [10]. This might help to understand which events and factors are more likely to cause distress to the animal. Among these, cortisol represents one of the most studied target molecules, due to the strict link between its synthesis and the state of physical and psychological stress of mammals [11].

1.2. Cortisol measurement

Cortisol is synthesized by the adrenal glands after the activation of the hypothalamic-pituitary-adrenal (HPA) axis [12]. The axis is activated daily and is responsible for a pulsatile hormone's secretion that follows diurnal and seasonal rhythms. The system is influenced by various external factors, such as feed intake, temperature and humidity, age, and physiological state [13]. In addition to the daily pattern, the HPA axis can also be independently activated by the sympathetic nervous system during circumstances that require an immediate expenditure of energy from a physical and mental point of view, such as a stress-related context. Due to the large number of cortisol receptors located in almost all tissues of the organism (nervous, musculoskeletal, immune, respiratory, cardiac, and reproductive), the hormone's secretion resulting from these situations can affect a number of basic physiological processes, with different consequences depending on the length of the stimulus [14]. In particular, the immune system is boosted by periods of acute stress and suppressed in association to a chronic secretion of the hormone [15], [16].

Cortisol measurement represents one of the key-topics of the recent literature on livestock welfare. Currently, one of the methods involves analysing the hormone's concentration in blood plasma, but the animal handling for the sampling collection itself induces a stress response enough to cause an immediate rise of the hormone's levels, altering the resulting data [17], [18]. Therefore, several studies aimed at finding new ways to obtain reliable measurements of the hormone's levels are being conducted [19]. To reach this goal, alternative, non-invasive matrices, such as faeces, milk and hair are being studied. However, all of them have been proved to present a limited correlation with the hormone's blood levels. In particular, hair and faecal cortisol are more indicative of a chronic stress situation and provide a reliable and valid reflection of long-term cortisol secretion [20], [21]. On the other hand, milk values are potentially representative of acute stress, but are influenced by a large number of factors that negatively affect the reliability of the related data [22]-[24]. In conclusion, these matrices do not allow us to identify potential punctual stressors.

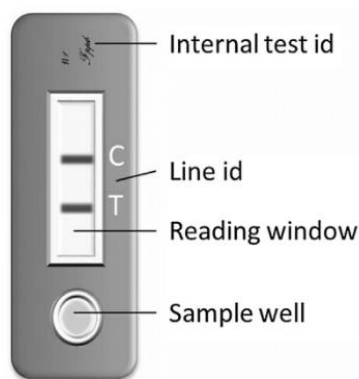


Figure 1. Scheme of LFIA device. Positions of the Test and Control lines are identified by initials printed on the cassette [27].

Recently, the cortisol's evaluation in saliva has drawn major attention as a non-invasive measure of stress. Salivary cortisol collection does not cause additional stress to the animal and samples result more representative of the underlying welfare status [25]. In addition, multiple studies report that salivary cortisol is indeed highly correlated with the plasma cortisol, of which it reflects the hormone levels' shift with a 30-minutes time-lag, thus allowing for more reliable results [18]. Because of these reasons, salivary cortisol has been extensively used to evaluate stress response in animals of different species [12], [26]. The availability of non-invasive diagnostic tools operable *in situ* would facilitate monitoring of animal welfare.

2. CORTICOW PROJECT

2.1. Hypothesis

The development of a rapid test capable of analyzing the salivary cortisol level of a bovine can provide an objective, non-invasive evaluation of the animal's acute stress contributing to the continuous improvement of livestock's welfare.

2.2. Aim

The aim of the CortiCow project is to develop and validate the on-field use of a Lateral Flow Immunoassay (LFIA) for the evaluation of acute stress in bovines. Several methods are available for salivary cortisol measurement in mammals, however rapid diagnostic tests for detecting salivary cortisol are confined to humans and, more recently, to dogs [27] (Figure 1). In cattle farming, this type of determination is already being explored, but the current measurement is performed using EIA (Enzyme ImmunoAssay) kits. This technique is precise, but expensive and exploitable only in a laboratory, while the animal farming science currently requires a screening, on field test, able to provide a qualitative answer (stressed/non stressed). These characteristics would allow the device to be operable *in situ* by non-trained personnel, ultimately facilitating the monitoring of animal welfare and supporting the farmer's management decisions in case of animals' acute stress.

2.3. Development

To achieve the proposed objective, the ongoing project involves two experimental phases: the first was carried out in March 2022 and was aimed at establishing the basal cortisol level for the test's sensitivity. The second phase started in March 2023 and is aimed at establishing the validity of the use of salivary cortisol in cattle's welfare evaluation, in order to ultimately set the basis for the development of an on-field, rapid test performing a qualitative determination.

2.4. Sample collection, storage, and preparation

In both phases the collection of saliva samples followed the same procedure. Saliva was collected by the SalivaBio Oral Swab device (Salimetrics, CA, USA). The swab was gently put under the animal's tongue and in the cheek pouches for 5-10 s (Figure 2). Saliva samples were collected from cattle in the morning (8:00 am - 9:00 am for the first phase; 4:00 am - 12.00 pm for the second phase); in order to minimize bias due to sampling, all samples were collected by a veterinarian who was trained for the purpose. All the analysis were performed at the Department of Chemistry of the University of Turin (Italy). Collected samples were transported to the laboratory at 4 °C and subsequently frozen to -20 °C and stored at the same temperature. For the analysis, samples were thawed at room temperature, centrifuged for 15 min at 2200 rcf and the resulting



Figure 2. Bovine saliva collection.

saliva extract was subjected to LFIA (first phase) and to both EIA and LFIA determination (second phase) without any further treatments.

2.5. First phase

Five female cows of Piedmontese breed aged between 2 and 10 years were involved in the first phase of the project. The cows were housed at the teaching farm of the Department of Veterinary Sciences, in Grugliasco (Turin, Italy). All animals were subjected to saliva samples collection in two consecutive days (Table 1) during which their daily routine was respected and lacking any known stress for the cows (e.g., none of the cows involved were subjected to veterinary checks nor movements). The cortisol's concentration determination was performed by comparing the samples in the studio with reference ones of known concentration. The comparison was carried out through the employment of an experimental LFIA, whose validity had been previously assessed. In accordance with the literature, all animals presented salivary cortisol levels lower than 4 ng/mL [28], ultimately chosen as the cut-off value for the future rapid test's sensitivity.

2.6. Second phase

The second phase of the Corticow project consisted in the on-field validation of the LFIA and the use of an EIA test as a reference method. The samples collection in this phase took place in a bull fattening commercial farm located in Savigliano (CN, Italy). Bulls come into the farm from multiple farms of origin located in France, and they are transported by a truck over an average distance of 800 km and average duration of transport of 9 hours. Transportation length and truck are in full compliance with the EU regulation on animal transport [4].

In this phase, we aimed to perform a biological validation of the LFIA, i.e. the measurement of the cortisol levels after a known biological stressor. We used the biological validation as an alternative to conducting an adrenocorticotrophic hormone

Table 2. List of bulls involved in the second phase of the project: group, bovine ids, date of birth, and breed.

Group	Bovine ID	Date of birth	breed
Test group	2243	29/03/2022	Charolaise
	2075	16/03/2022	Charolaise
	2217	27/02/2022	Charolaise
	2274	01/01/2022	Charolaise
	2725	27/12/2021	Mixed breed
	2732	26/12/2021	Mixed breed
	2735	01/02/2022	Charolaise
	2388	30/03/2022	Charolaise
	2386	22/03/2022	Charolaise
	2246	16/04/2022	Charolaise
Control group	3702	17/01/2022	Charolaise
	3997	28/02/2022	Limousine
	3691	17/11/2021	Limousine
	3707	02/02/2022	Charolaise
	3681	19/09/2021	Limousine
	3995	19/02/2022	Limousine
	2814	19/10/2021	Limousine
	3700	31/12/2021	Limousine
	7407	14/10/2021	Limousine
	4204	24/09/2021	Limousine

(ACTH) challenge. The administration of ACTH induces synthesis and release of adrenocortical glucocorticoids (GSs), including cortisol [29]. However, the use of a biological stressor (e.g., capture and restraint, social tension, and transport) is recommended since this test ensures that the method will appropriately measure concentrations of GCs comparable to those normally detectable in animals exposed to genuine stressors [30], [31].

We collected the saliva samples after the transport of the animals, a well-known stressor in cattle [32]. A total of 20 bulls of different breeds and aged between 11 and 18 months were involved in this phase (Table 2). The test group was composed of 10 newly arrived bulls (i.e., exposed to the transport stress), while the control group was composed of 10 bulls housed in the farm for at least 60 days. The daily routine of the control group was respected, and the bulls were not subjected to a known stress in the days before the sampling.

The sampling protocol timeline of this phase is shown in Figure 3. Briefly, saliva samples were collected from the test group at the arrival, and the collection continued during the subsequent 8 hours at six different time intervals, in order to evaluate the cortisol level's fluctuation through time. Saliva samples were collected from the control group at three different time intervals to compare the cortisol levels of animals already

Table 1. Bovine IDs, date of birth, and saliva collection time in the first phase of the project.

Bovine ID	Date of birth	Saliva collection time on the first day (31/03/2022)	Saliva collection time on the second day (01/04/2022)
5072	28/11/2018	8:35	8:27
0292	17/03/2010	8:38	8:30
0638	11/05/2020	8:40	8:32
0636	29/02/2020	8:42	8:33
2410	16/09/2016	8:46	8:36

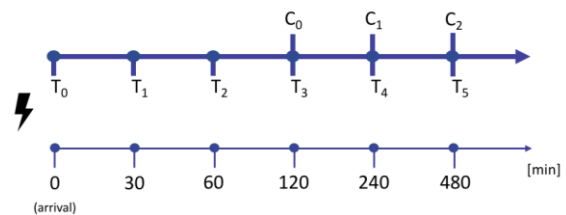


Figure 3. Sampling protocol timeline of the second phase of the project. T = test group; C = control group.

The bottom line indicates the time of the sample collection.

The above line indicates the group of animals involved in the sampling.

present on the farm with those of the newly arrived animals. We collected a total of 90 samples, 60 from the test group and 30 from the control group. The saliva samples were immediately stored at 4 °C and sent to the laboratory for the analysis.

Moreover, we planned to repeat the same sampling protocol after 60 days from the arrival of the test group: on that day, the bulls belonging to the previous test group will become the control group, and the newly arrived bulls will become the test group.

3. CONCLUSIONS AND EXPECTED IMPACTS

The comparison between animals subjected to stress (test group) and the non-stressed (control group) will determine if the salivary cortisol concentration can be used to evaluate the state of stress of a bovine, and to which extent. We expect to highlight a correlation between the two factors in the study, thus posing the basis for the subsequent development of a Lateral Flow Immunoassay. The device will be able to perform the same discrimination based on the cut-off value defined in the first phase of the trial.

The availability of this non-invasive diagnostic tool operable *in situ* will facilitate monitoring of animal welfare. In the short term, this innovation is expected to assist farmers and veterinarians in performing a more objective evaluation of the animal's acute stress levels.

In the long term, the device could become a key-instrument for the EU's growing necessity to better monitor and identify the potential stressors in animal farming. This urgency arises from various factors: firstly, the increasing diffusion of intensive farming systems, which often represent a cause of concern for the general public in relation to the animals' welfare and thus lead to an increasing pressure on the main Institutions. The animals' growing densities in farms represent a concern for the veterinarians as well, since this phenomenon furtherly reduces the available visiting time per animal, ultimately leading to the necessity of identifying objective parameters that can be evaluated easily, preferably by the farmer itself.

Lastly, the EU legislation is promoting the identification of measures that relate the animals' health and welfare status, further allowing the application of a clinical approach based on prevention rather than treatments [33]. Due to these reasons, the development of this device could further enable and promote the application of animal well-being strategies, leading to an overall increase in the animals' welfare conditions.

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