Validation of a qualitative immunochromatographic test for the noninvasive assessment of stress in dogs

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VALIDATION of a QUALITATIVE IMMUNOCHROMATOGRAPHIC TEST for the NONINVASIVE ASSESSMENT of STRESS in DOGS

F. Di Nardo[1], L. Anfossi[1*], L. Ozella[2], A. Saccani[3], C. Giovannoli[1], G. Spano[1], C. Baggiani[1]

[1] Department of Chemistry, University of Turin. Via Giuria, 5. I-10125 Turin, Italy

[2] Department of Life Sciences and Systems Biology, University of Turin. Via Accademia Albertina 13. I-10123 Turin, Italy

[3]EuroClone SpA, Via Figino, 20/22. I-20016 Pero, Milan, Italy

* to whom correspondence should be addressed. Tel: +390116705219, fax: +390116705242. E-mail: laura.anfossi@unito.it

Abstract

Salivary cortisol is regarded as a reliable parameter for the noninvasive assessment of the welfare of animals, because it is strictly related to stress levels. Several methods are available for salivary cortisol measurement in mammals, however rapid diagnostic test for detecting salivary cortisol are confined to humans. The availability of such non invasive diagnostic tools operable in situ would facilitate monitoring of animal welfare. The Cortisol stress™ test provides a simple and rapid tool to discriminate cortisol levels in canine saliva above or below 4 ng/ml, which has been suggested as the cut-off value for distinguishing unstressed dogs from those experiencing stress. The test is based on a competitive immunochromatographic assay (ICT) using gold nanoparticles as probes, in which the color intensity of the Test line is inversely correlated to the salivary cortisol level. The qualitative result is obtained by the visual observation of the color formed on the Test line compared to that of the Control line.

We evaluated the accuracy of the test by determining salivary cortisol in 85 samples of canine saliva belonging to dogs with very variable age, sex, breed, and life history, and comparing the qualitative results to those obtained by a reference ELISA kit. Agreeing results were obtained through the two methods, and the ICT showed high diagnostic sensitivity, specificity and efficiency (100%, 98.4%, and 98.8%, respectively). Furthermore, we evaluated the precision of the test by an experimental design approach, which combines errors due to within-day and between-day variation with the biological variability, and demonstrated that the test could be reliably applied for correctly classifying canine samples, according to their salivary cortisol level. Moreover, we studied the shelf-life of the device in three experimental conditions. We confirmed the stability of the ICT at 4°C and 25°C for at least six months and observed similar results for an accelerated stability study conducted for 7 days at 37°C, which suggest that the stability of ICT device could be estimated by the accelerated experiment alternatively to the real-time study.

Keywords

Saliva, cortisol, lateral flow immunoassay, shelf-life.

1. Introduction

There is a growing social interest in the well-being and improvement of living conditions for domesticated animals due to ethical considerations and evidence showing that stress can impair immune function in animals, making them more susceptible to disease including susceptibility to infection [1].

Accordingly, the European Union recognizes as a priority the welfare of animals and has developed specific legislation to animal welfare for improving the quality of animals' lives in accordance with citizens' expectations and market demands [2]. However, concerns about living conditions of animals must align with economical considerations. Efforts to improve animal care and management should be driven by scientific

investigations, as well as social perceptions. Discussion of animal welfare will become more objective and meaningful by development of methods that help establish an animal's state of welfare. Welfare refers principally to the subjective psychological state of the individual and stress is the term used to describe environmental factors eliciting adaptation mechanisms in the subject and the subject's response to these challenges [3]. As we are not able to know directly an animals' psychological state, we require indices that correlate with stress.

An accepted approach to the study of stress and welfare in animals is measuring hypothalamic-pituitary-adrenal (HPA) axis activity through the determination of plasma cortisol levels [3]. However, blood collection is an invasive procedure that requires skilled technical capabilities and has been shown to act as a source of stress itself. Several alternative methods such as the measurement of the hormone in saliva, urine, feces or hair have been developed to overcome the stress induced by blood sampling [3-6] and to allow repeated measurements [7]. Because salivary cortisol is highly correlated with plasma cortisol and saliva sampling is a noninvasive practice, salivary cortisol has been extensively used to evaluate stress response in animals of different species [4-5,8].

Several behavioral indicators of stress have been identified and applied to study the level of acute and prolonged stress in dogs [9]; nevertheless, salivary cortisol is most commonly used for assessing stress in these animals and has been used in studies of dogs' welfare, reaction to stress challenge, and human-animal interactions [7, 10-14]. Radio- or enzyme-immunoassays are almost exclusively employed for this purpose, either as in-house validated assays [14] or, most frequently, as commercially available kits [7,9,11-13].

The Cortisol stress™ test by Camon SpA is an immunochromatographic test aimed at qualitatively evaluating cortisol levels in saliva of dogs. It has been specifically developed as a simple and accurate method to evaluate the stress experienced by dogs. These animals are the most common companion animals and are used also as working animals (e.g. as guide dogs, police dogs, etc.) and in medical or educational facilities (animal-assisted activities, animal-assisted education, or animal-assisted therapy). Therefore, the periodic evaluation of stress in dogs is important to monitor their well-being and, mostly, to provide adequate security to people with whom they come into contact.

Among rapid diagnostic methods, the immunochromatographic strip test (ICT) technology is attracting a growing interest for veterinary applications, mainly because it allows very rapid, simple, *in situ* analyses to be carried out. In this context, the Cortisol stress™ test provides a tool for the assessment of stress in dogs, executable outside the laboratory by non-trained personnel, and without the need of any equipment. It is intended for discriminating stressed animals on the basis of the salivary cortisol above a defined cut-off value, which has been set at 4 ng/ml. The point identified for differentiating between stressed and non-stressed animals is in agreement with data available in the literature regarding salivary cortisol concentrations measured in dogs under basal conditions. These generally are reported to fall into the range of 0.2 – 3 ng/ml [8 and the references therein]. Beerda and co-workers measured a mean basal level of 2.2 ng/ ml in ten dogs

differing in breed, age and sex. They observed a significant increase of salivary cortisol in response to several types of acute stress, ranging from 4.7 to 36 ng/ml depending on the stress stimulus applied, with mean levels of approximately 6 ng/ml [7]. Buttner et al. reported basal levels between 2.5 and 3.5 ng/ml for dogs involved in a competition and observed a slight increment of cortisol levels after the competition [14]. Hekman et al. recognized a clear inflection point in the distribution of salivary cortisol of 28 healthy dogs and accordingly classified animals into two groups named "lower cortisol levels" and "higher cortisol levels", based on their salivary cortisol being below or above 6 ng/ml [9]. However, according to authors' conclusions, the tentative to correlate cortisol levels to animal distress assessed through behavioral observations, was inconclusive.

It is worth mentioning that several factors have been reported to affect basal levels of salivary cortisol, such as the living site, the size of the animal and the neutering condition [12]. Conversely, other sources of biological variability (such as the animal's age and gender, as well as the time of the day and the location of the collection) have been reported as not affecting salivary cortisol concentrations [11, 12] [11].

Accepting some uncertainty on the appropriateness of the cut-off value, our intent for conducting this work was to validate the Cortisol stress™ test as far as: (i) its ability to accurately distinguish cortisol levels above or below the declared cut-off value by comparing results obtained on canine saliva samples from the ICT with those obtained by a reference immunoenzymatic test for a representative group of dogs of different ages, sex, and breed; (ii) its precision, as evaluated by an experimental design approach, previously proposed by Lattanzio et al. and adapted here to consider casual errors as the sum of within-day, between-day, and biological variability [15]; (iii) the shelf-life of the immunochromatographic device. This has been assessed by means of real-time and accelerated stability experimentswith the further aim of comparing results obtained at different temperatures and verify if accelerated stability experiments could have predictive value for the real-time shelf life of ICT devices.

The Cortisol stress™ is the first rapid test described for the measurement of salivary cortisol in dogs. Immunochromatographic tests to determine cortisol in saliva of humans have been described [16-17], however they exploit enzymatic reactions to produce the signal and need several steps and specially designed optical readers to obtain the results. An ICT device has been proposed for veterinary applications that allowed stress assessment in pigs through measuring salivary cortisol [18]. The test is semi-quantitative, however it requires reading the optical density of colored lines to provide the results. Therefore, to the best of our knowledge, this is the first report describing a completely stand-alone device for the detection of cortisol in saliva.

2. **Materials and methods**

2.1. Materials

Cortisol Stress™ was obtained from Camon SpA (Italy). The reference ELISA (Enzyme-Linked ImmunoSorbent Assay) kit used was the Cortisol ELISA kit - Salimetrics assay 1-3002 (Salimetrics, CA, USA).
Cortisol powder was obtained from Sigma-Aldrich (MO, USA). The cortisol stock solution (2 mg/ml) was prepared in absolute ethanol and stored at -20°C. Standard solutions were prepared daily by properly diluting the stock solution.
Statistical calculations were carried out with SigmaPlot 11.0 software.

2.2. The ImmunoChromatographic Test

Tests to determine cortisol levels were carried out at room temperature, by applying 60 µl of cortisol standard solutions or saliva samples to the sample well of the ICT device, waiting for 10 minutes and recording results. The results were both qualitatively estimated by the naked-eye and quantitatively evaluated by acquiring the images (OpticSlim 550 scanner, Plustek Technology GmbH, Norderstedt, Germany) and measuring the intensity of the color on each line with the QuantiScan 3.0 software (Biosoft, Cambridge, UK).

2.3. Characterization of the ICT for detecting cortisol in canine saliva

The limit of detection (LOD) of the test was evaluated by applying standard solutions of cortisol (0, 0.5, 1, 2, 4, 8, and 16 ng/ml) diluted in phosphate buffer with 0.5% BSA and 0.1% Tween 20 (running buffer) to the ICT cassette. Each standard was measured in triplicate and results, expressed as the ratio between the color intensity of the Test line (T) divided by the color intensity of the Control line (C), was plotted against cortisol concentration. Data were analyzed by a nonlinear regression using the four-parameter logistic equation and LOD was calculated as the cortisol concentration corresponding to the signal of the blank minus three standard deviation of the blank. The dynamic range was considered as the cortisol levels giving signals between 10% and 90% of the signal of the blank.
The selectivity of the test towards other natural and synthetic steroids was determined by the manufacturer as the cross-reactivity values (calculated as the corticosteroid concentration that gives 50% inhibition of the signal of the blank divided by the cortisol concentration that gives 50% inhibition of the signal of the blank x 100) and resulted as follows: dexamethasone 20%, 11-deoxicortisol 0.9%, prednisolone 5.6%, corticosterone 0.6%, 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, testosterone, estradiol, and estriol < 0.1%, danazol < 0.01%.
We studied the influence of matrix composition on the assay by pooling three saliva samples, which cortisol content was previously measured by the ELISA reference method and resulted to be lower than 1 ng/ml. Pooled saliva was fortified with cortisol at four levels: 1, 2, 4 and 10 ng/ml.

2.4. Validation of the ICT for the qualitative analysis of salivary cortisol

The imprecision of the ICT was considered to be due to the sum of 3 components: the within- and between-day variations due to the test, and the biological variability. We estimated this parameter by an experimental design approach, previously proposed by Lattanzio et al. [15]. However, we adjusted the number of experiments according to the low amounts of saliva sample available for each subject. Therefore, the study was conducted by analysing 4 sets of canine saliva, previously tested by the reference ELISA and showing the same cortisol level. The samples were analysed on three days. On each day, two samples were analysed in duplicate and two were analysed singly, as schematized in Figure 1. In this way, precision was calculated as CV% on a total of 18 replicate measurements.

We evaluated the accuracy of the test by analyzing 85 samples of canine saliva. The tests were carried out as described above. After the immunochromatographic run, we observed the color intensity developed on the Test and Control line by the naked eye and compared them. The criterion for judging the result of the ICT was defined as follows: if the color intensity of the Test line was higher than that of the Control line the sample was assigned as negative. Conversely, when the color intensity of the Test line was equal to /lower than that of the Control line, the sample was assigned as positive.

Each sample was run once and the visual result was evaluated by three different subjects.

2.5. Sample collection, storage and preparation

A total of 60 healthy dogs were involved in the study (Table 1). Saliva was collected once for each subject, except for 5 dogs, which were sampled twice at different times (May and November 2015).

Saliva was collected by the SalivaBio Children's Swab (Salimetrics, CA, USA). The swab was gently put under the dog's tongue and in the cheek pouches for 60 s. Saliva samples were taken from participants between 11 a.m. and 4 p.m. In order to minimize bias due to sampling, all samples were collected by a veterinarian and a professional dog trainer, who were trained for the purpose.

Collected samples were immediately frozen to -20°C and stored at the same temperature. For the analysis, samples were thawed at room temperature, centrifuged for 15 minutes at 4000 rpm and the resulting saliva extract was subjected to both ELISA and ICT determination without any further treatments.

2.6. ELISA for salivary cortisol

According to the manufacturer, the reference ELISA was intended for measuring cortisol in saliva of humans and some animals. Although the assay had not been validated for measuring salivary cortisol in dogs, it has been widely and successfully applied for measuring cortisol levels in canine saliva in several previous works [10, 19]. According to the claim of manufacturer, the assay shows the following figures of merit: within- and between-assay precision below 7 and 11%, respectively; recovery comprised between 97 and 113%; lower limit of sensitivity (calculated as the concentration corresponding to the signal of the blank minus two standard deviation of the blank) of 0.07 ng/ml. Cross-reactivity assessed towards several other

corticosteroids and hormones showed negligible values, except for dexamethasone (cross-reactivity 0 19%)) [20].

Salivary cortisol was measured following manufacturer's instructions [20].

2.7. Stability of the ICT device

With the aim of evaluating the shelf-life of the developed ICT device, we performed real-time and accelerated stability experiments [21]. For the accelerated stability experiment, we kept ICT strips at 37°C for 7 days and tested them on day 0, 1, 3 and 7. For the real-time stability experiment, we keep ICT strips at room temperature and at 4°C, respectively, for 6 months, and tested them on week 0, 1, 3, 6, 12, and 24. For each experiment, we used cortisol standard solutions at 1 ng/ml (negative control) and 6 ng/ml (positive control). For all experiments, strips were stored in the dark and with dessicant added.

3. Results and discussion

3.1. Analytical parameters of the Cortisol stress™ ICT

The Cortisol stress™ is a qualitative immunochromatographic test that uses gold nanoparticles (GNPs) as colored probes for the visual interpretation of results. GNPs are conjugated to the anti-cortisol antibodies and included in the device together with a strip composed of: the nitrocellulose membrane (onto which two lines of bio-reagents are provided to allow assay completion), the sample pad, and the adsorbent pad. The strip is included into a plastic cassette furnished with a sample well and a reading window (Fig.2). The test is designed as an indirect competitive immunoassay; the application of the sample promotes the solubilization of the GNP-labeled anti-cortisol antibodies and their flowing across the strip. During the flow, GNP-labeled antibodies bind to the analyte present in the sample. The two reactive zones on the nitrocellulose membrane comprise a competitor bio-reagent that binds to the free GNP-labeled antibodies (Test line) and a capturing bio-reagent that captures any GNP-labeled antibodies (Control line). Therefore, in the absence of the target analyte, the GNP-labeled antibodies bind to both reagents forming the Test and Control lines so that two colored lines appear. Conversely, if sufficient amount of the analyte is present in the sample, GNP-labeled antibodies are bound to the analyte and cannot interact with the competitor reagent on the Test line causing the appearance of the color only on the Control line zone. Within these two extremes, the color of the Test line progressively fades as the amount of the analyte in the sample increases. Contemporary, the color of the Control line intensifies, because GNP escaped from the Test line are captured there. According to manufacturer's instructions, the color of the Test line is more intense than the color of the Control line for cortisol levels in canine saliva below 4 ng/ml (i.e.: the cut-off value). Observing a color of the Test line equal to or less intense than the color of the Control line means that the salivary cortisol levels have reached or exceeded the cut-off value (Fig. 3a).

Although the ICT is intended for the qualitative assessment of cortisol levels in canine saliva, we estimated the limit of detection (LOD) and the dynamic range of the assay by using standard solutions of cortisol diluted in buffer (Fig. 3b). The intensity of the color on each line was measured by scanning images of the devices after the run and the measured color for the Test line (T) divided by the measured color of the Control line (C) was plotted towards cortisol concentrations. The $IC_{50,}$ LOD, and dynamic range were calculated to be 0.7 ng/ml, 0.2 ng/ml and 0.2-11 ng/ml, respectively. According to manufacturer instructions, the visual interpretation of the test would give positive results (i.e.: cortisol levels above 4 ng/ml) if the color of the Test line is equal or less intense than that on the Control line. That would mean an expected T/C ratio ≤1 for cortisol levels above 4 ng/ml. The cortisol amount corresponding to the cut-off value gave a T/C ratio just above 1, when instrumentally recorded. However, as the Test line is less focused than the Control line, the naked perception slightly differed from the instrumental measure of line intensity. The area of the peak measured by the software for the Test line resulted generally higher than the color perceived by the naked eye, which is more sensible to the absolute intensity of the color rather to the integral over the area.

We also estimated matrix interference on the assay by measuring cortisol in a saliva sample obtained by pooling three individual samples to mitigate biological variation. We determined the cortisol content of the pooled saliva sample by the reference ELISA kit and generated matrix-matched calibrators by fortifying the pooled sample with known amounts of cortisol. We compared results obtained on the matrix-matched calibrators to those obtained by testing calibrators diluted in the running buffer (Figure 3b) and observed an incomplete overlap between the two curves, which means that some matrix interference existed. Nevertheless, the qualitative interpretation of results was not affected in a significant way, since the T/C ratio for the pooled saliva fortified at 4 ng/ml was still about 1, as desired.

3.2. Validation of the ICT for the qualitative assessment of salivary cortisol in dogs

The validation of the ICT involved evaluating its precision, according to a strategy proposed by Lattanzio et al. in 2013 [15] for the validation of immunochromatographic tests, which permits taking into account three different sources of errors, namely the within-and between-day variation and the biological variability. The first two components are attributable to the analytical method employed, while the third is independent from the method, however extremely important in the evaluation of biological parameters on which the influence of the biological variability is unknown or not completely clarified. Lattanzio et al. first applied this approach to assess the precision of an immunochromatographic assay for measuring a mycotoxin in cereals. We necessarily modified the original protocol to meet restrictions due to limited availability of sample, mainly in terms of the volume of saliva collected for each individual. Therefore, we tested samples belonging to four dogs, which had been found to have comparable levels of cortisol according to the reference ELISA kit (estimated cortisol levels: 1.7, 1.8, 1.9, 1.9 ng/ml). The experiments were repeated on three days and each sample was measured in single or in duplicate on different days, as schematized in Figure 1.

Since we expected biological variability to affect overall imprecision in a larger extent compared to method repeatability, we calculated the mean values of the signal (T/C) obtained as measurements for each individual dog on the various days (n=4) and compared the mean values of each dog by a one way analysis of variance (ANOVA). We obtained that there was a statistically significant difference (P=0.004) among the groups, with a power of the performed test above the desired value (power of performed test with alpha=0.050: 0.893). Comparing the results belonging to the four dogs by all pairwise multiple comparison procedures (Holm-Sidak method), we found that the sample belonging to one dog gave a mean T/C value significantly different from the others (comparison for factors: dog#1 vs dog#22 P<0.001, significant level P=0.009, significantly different; dog#1 vs dog#3 P=0.006, significant level P=0.010, significantly different; dog#1 vs dog#4 P=0.029, significant level P=0.013, not significantly different). Thus we decided not to consider data obtained on dog#1 sample for calculating the overall precision. The other three samples showed no significant differences among their T/C mean values. Therefore, we calculated an overall mean value for the signal ratio (T/C) of 3.70 and a relative standard deviation of 13.5%, based on n=14 experiments. Due to the limited numerosity of the sets we could not attribute the source of errors to a specific component within the three listed above. We could hypothesize that there is not a statistically significant difference among mean values obtained on different days (P = 0.391), although the power of the one way ANOVA was below the desired power for this comparison.

The observation that one sample within the four involved in the study provided results significantly different from those of the other three suggested that the biological matrix could have a strong influence on the ICT capability of correctly measuring salivary cortisol in dogs. It should be noticed that we used saliva samples belonging to four individuals without applying any exclusions or partitions, except for the level of cortisol measured by the reference ELISA kit.

Also for the following assessment of the accuracy of the test, we did not apply any exclusion or partition criteria. On the contrary, we recruited individuals as different as possible as far as the age, gender, breed and life history, with the aim of evaluating the general applicability of the test.

We involved 60 dogs in the study, which biological parameters are resumed in Table 1. Saliva was collected once for each individual, except for 5 dogs, which were sampled twice. Therefore, a total of 65 canine saliva samples were collected and their cortisol content was determined by the reference ELISA method. Accordingly, 7 samples were assigned as positive (salivary cortisol ≥ 4 ng/ml, mean salivary cortisol 7.0 ng/ml, with levels comprise between 4.0 and 19.1 ng/ml) and 58 as negative samples (salivary cortisol < 4 ng/ml, mean salivary cortisol 2.0 ng/ml, with levels comprise between 0.9 and 3.9 ng/ml). Additional 20 samples were generated by fortifying negative samples with known amounts of cortisol (2.5, 5 and 10 ng/ml) to obtain further 16 positive and 4 negative samples.

Hence, a total of 85 samples were tested by the Cortisol stress™ ICT, including raw and fortified samples. The visual result was assessed by three different operators, who observed the strips by the naked eye 10 min

after the application of the sample. According to the definition of the ICT result as positive/negative based on comparing color intensities of the Test and Control lines, we classified 25 samples as positives and 60 as negatives. None of the samples gave uncertain results, and the judgments of the three subjects involved in the study were always in agreement.

From these results we obtained the figures of merits for the validation of the qualitative test [22-23] that are summarized in Table 2. In particular, we calculated: (i) the diagnostic sensitivity of the test (Se), defined as the rate of truly positive results; (ii) the diagnostic specificity of the test (Sp), defined as the rate of truly negative results; (iii) the efficiency of the test, defined as the fraction of tests correctly classified; (iv) the predictive value of positive test (PV+) and the predictive value of a negative test (PV-), defined as the probability that the subject that obtained a positive or negative test result has the given positivity or negativity, respectively [22]. The Cortisol stress™ ICT demonstrated very high diagnostic sensitivity and specificity, thus confirmed its wide applicability for assessing the level of salivary cortisol in dogs, independently on the age, gender, breed and life history of the animal.

3.3. Shelf-life study

Stability is a crucial feature for ICT devices mainly because they are especially intended for on field use and hopefully should not require special storage conditions. However, they contain all materials and bio-reagents (antibodies, proteins, etc.) needed for the assay and most of these components are known to be intrinsically sensitive to environmental conditions. Some authors have evaluated the real-time stability of ICT strips, which rarely achieved shelf-life acceptable for commercial purposes (6-12 months) [23-24]. On the other hand, a major limitation of shelf-life studies is represented by the fact that the evaluation of the real-time stability for such long periods of time is often inconvenient. More frequently, accelerated stability experiments have been conducted by keeping ICT devices at high temperatures (typically: 37°C or 60°C) for short periods of time (7-21 days) and if no significant decay was observed during the test period, the result was converted to a real time stability of months [25-26]. The suitability of this conversion had been derived (not without a certain recklessness) from consolidated stability studies on microwell-based immunoassays.

In this work, we conducted the study on the ICT real-time stability in two different storage conditions: by keeping the ICT device in the refrigerator (4°C) and at room temperature, without any additional precautions, except for those included in the manufacturer packaging (each device was individually packed, with light protection and in the presence of a dessicant). We verified the stability of the Cortisol stress™ ICT for six months at both temperatures considered, without observing significant differences among them. In details, a positive and a negative control could be correctly attributed, based on the visual interpretation of the colour intensity of the lines, even after six months of storage. Also, the quantitative analysis of signals confirmed the visual observation (Figure 4a). Although we observed a slight decreasing trend of the T/C ratio for both controls, which was more evident for the devices stored at 4°C, we concluded that the ICT is

acceptably stable for six months and does not require a specific storage temperature. The observed decrease of the T/C ratio was due principally to a fainter T-line, which was more evident at 4°C. The effective long-term stability shown by of the Cortisol Stress™ device is uncommon for a rapid test based on competitive immunoassay formats [23-25], while essential for its on-field application. Comparable shelf-life have been obtained for ICT diagnostics based on two-sites immunometric assays [26], in which bio-reagents are provided in large excess compared to the analyte amount.

Moreover, we carried out an accelerated stability experiment by keeping the ICT device at 37°C for one week. Also in this case, we obtained results acceptably comparable to those of the freshly prepared device (Figure 4b). The profiles of the T/C ratio plotted towards the time of storage are qualitatively similar for the real-time and the accelerated stability studies. The percentage decrease in terms of T/C ratio measured at day 7 for the accelerated stability experiment and at month 6 for the real-time stability experiment were 81% and 83% for the negative control, and 80% and 81% for the positive control, respectively. This finding indicates that the accelerated stability test at 37°C could represent a feasible alternative for estimating the real-time shelf-life of ICTs.

4. Conclusions

In this work, we evaluated the reliability of the immunochromatographic test Cortisol stress™ as a tool for the noninvasive assessment of stress in dogs. The test measures salivary cortisol and provide a yes/no response, which depends on the level of cortisol compared to the cut-off value established to distinguish animals experiencing stress from those not affected by persistent or chronic stress. We evaluated the reliability of the test by comparing the qualitative results provided by the ICT with the cortisol level measured by a reference ELISA kit on 85 samples of canine saliva. From these data, we calculated excellent figures of merits for the qualitative test, i.e.: the diagnostic sensitivity and specificity were 100% and 98.4%, respectively, while the positive and negative predictive values were 95.8% and 100%, respectively. It is worth noticing that we involved 60 different dogs in this accuracy assessment, largely differing in age, sex, breed and life history. About 20 of the sampled individuals were recruited in the city kennel, almost 30 belonged to a local shelter and the minority was house dogs. Moreover, we considered the reproducibility of the output given by the test by measuring the intensity of the color of the two sensitive lines that are used to establish the level of cortisol in the saliva and, as a consequence, to classify the individual as stressed or not. The measure was repeated to take into account within and between-day variations and biological variability. We concluded that the precision of the test is adequate to allow the correct classification of samples, though we found that biological variability could have an impact on color intensity of the lines.

Finally, we studied the long term stability of the device and demonstrated that it could be stored at room temperature for at least six months.

Ethical considerations

The study was based on voluntary participation, and the aims and sampling procedures were disclosed to the owners, shelter managers, and kennel officers. Informed consent was obtained before any procedure. All procedures were performed in respect of the Italian legislation on animal care (Legislative Decree 2014/26 implementation of EU Directive 2010/63/EU).

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Figures

Figure 1. Scheme of the experimental design for estimating ICT precision.

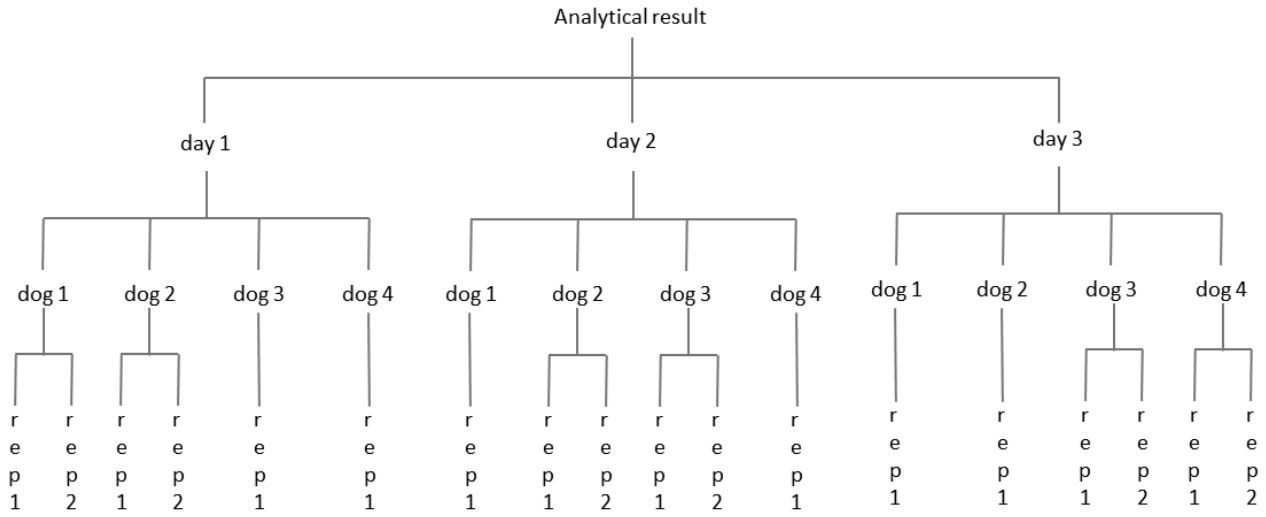


Figure 2. Scheme of the ICT device after sample running: the strip is included into a plastic cassette providing a sample well and a reading window. Positions of the Test and Control lines are identified by initials printed on the cassette.

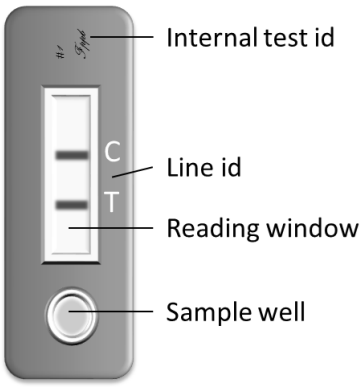
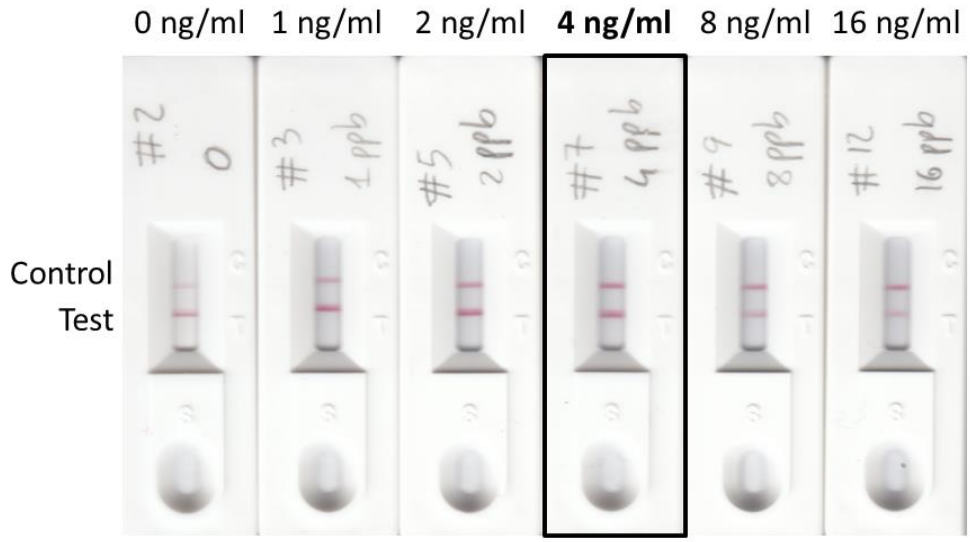


Figure 3. Standard curve for measuring cortisol by the ICT carried out by diluting cortisol in the running buffer. Images of the strips: the color intensity of the Test line progressively diminished as the cortisol concentration increased (T > C for cortisol levels beyond 4 ng/ml, T < C for cortisol levels above 4 ng/ml) (a) For quantification, the color of the T-line (normalized by the color of the C-line) is plotted against cortisol concentration in logarithmic scale (b, open circles). The triangles represent a pool of three samples of canine saliva fortified at different levels of cortisol to evaluate matrix effect.

a



b

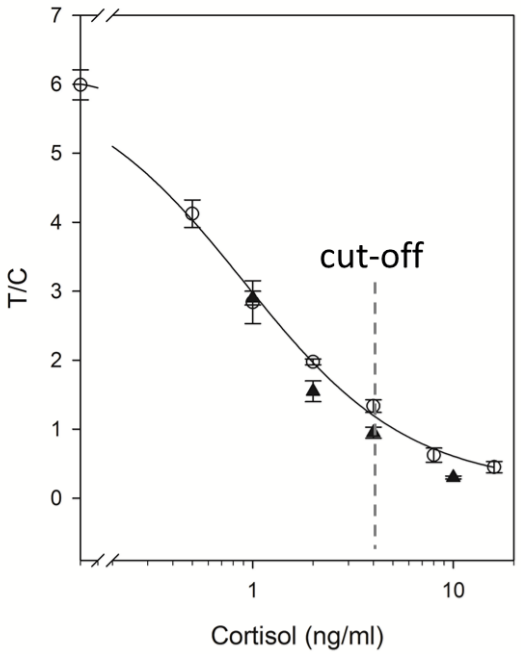
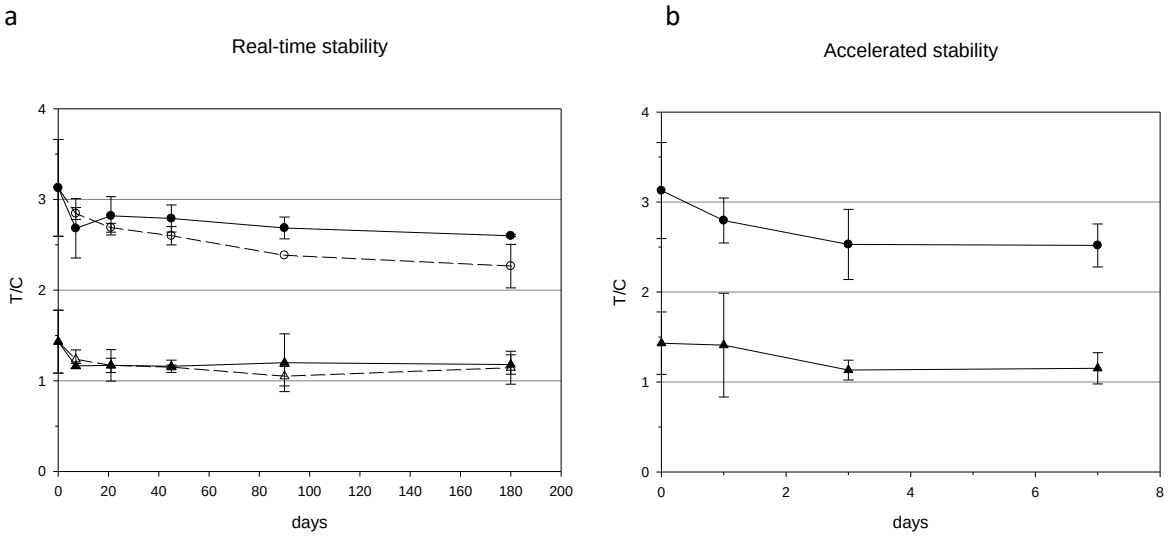


Figure 4. Shelf-life of the ICT device as measured in (a) real-time and (b) accelerated experiments. Circles represent the negative control and triangle the positive control. Open symbols were used for the experiment conducted at 4°C in the real-time stability test, while solid symbols shows data obtained for the room temperature experiment. Bars represent standard deviations (n=2)



Tables

Table 1. Description of the 60 dogs involved in the study.

Sex		Age			Breed (number of subjects)
M	F	<2	2-8	>8	German Sheperd (3), Beagle (3), Dogo Canario (5), French Bouledogue (3), Pit bull (9), Jack Russel (2), Dachshund (2), Australian Cattle Dog (3), Basset Hound (3), Dobermann (2), Collie (1), Rottweiler (1), Mixed-breed (24)
29	31	10	41	9	

Table 2. Figures of merits for the qualitative ICT, calculated with the cut-off level at 4 ng/ml of salivary cortisol.

Se TP/(TP+FN)	Sp TN/(TN+FP)	PV+ TP/(TP+FP)	PV- TN/(TN+FN)	Efficiency (TP+TN)/(TP+FP+TN+FN)
100%	98.4%	95.8%	100%	98.8%

Se = number of truly positive tests divided by the sum of known positive samples
Sp = number of truly negative tests divided by the sum of known negative samples
PV+ (PV-) = number of truly positive (negative) tests divided by the sum of positive (negative) tests
Efficiency = number of results correctly classified divided by the total of tests done.