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Original Article

Evaluation of adenosine deaminase in saliva and serum, and salivary α-amylase, in canine pyometra at diagnosis and after ovariohysterectomy

F. Tecles[a], D. Escribano[b], M.D. Contreras-Aguilar[a], C.P. Rubio[a], M. Szczubiał[c], J.J. Cerón[a], R. Dąbrowski[c,*], A. Tvarijonaviciute[a]

[a] Interdisciplinary Laboratory of Clinical Analysis of the University of Murcia (Interlab-UMU), Regional Campus of International Excellence 'Campus Mare Nostrum', University of Murcia, Campus de Espinardo s/n, 30100 Espinardo, Murcia, Spain
[b] Department of Animal and Food Science, Faculty of Veterinary Medicine, Universitat Autonoma de Barcelona, 08193 Bellaterra, Spain
[c] Department and Clinic of Animal Reproduction, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-612 Lublin, Poland

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ABSTRACT

An assay for adenosine deaminase (ADA) was validated in serum and saliva in dogs. Changes in ADA and salivary α-amylase activities were analysed in 26 bitches diagnosed with pyometra and compared with activities in 19 healthy bitches. All animals were classified according to the American Society of Anaesthesiologists (ASA) scoring for physical status. In the validation study, the ADA assay had an imprecision < 12% and determination coefficients > 0.90 in linearity under dilution experiments, with recoveries of 99.2–114.4%. On the day of presentation, salivary ADA activity was significantly higher in dogs with pyometra than in healthy dogs (median values 7.1 IU/L vs. 0.8 IU/L, respectively; $P < 0.01$). ADA had a moderate positive correlation with leucocyte and band neutrophil counts, haptoglobin, salivary α-amylase and ASA score, and a low positive correlation with C-reactive protein. There were no significant differences in salivary α-amylase activity between dogs with pyometra and healthy dogs (57.3 IU/L vs. 27.4 IU/L, respectively). Salivary α-amylase had a low correlation with ASA grade, and leucocyte and band neutrophil counts. In 7/26 bitches with pyometra that were sampled 3 and 10 days after ovariohysterectomy, there were no significant changes in α-amylase or ADA activities. These results indicate that ADA activity is increased in the saliva of bitches with pyometra, probably related to systemic inflammation.

Introduction

Pyometra is relatively common in intact bitches and its diagnosis is aided by ultrasonography (Bigliardi et al., 2004), along with measurement of acute phase proteins and leucocyte (white blood cell, WBC) counts; assessment of these parameters during recovery may be helpful for monitoring systemic inflammation and for detecting early complications (Dąbrowski et al., 2009; Jitpean et al., 2014). Saliva can be used for evaluation of markers of inflammation and stress, and can be collected relatively non-invasively and with minimal stress in veterinary species, including dogs, for the evaluation of markers of inflammation and stress (Vincent and Michell, 1992; Parra et al., 2005).

Adenosine deaminase (ADA; Enzyme Commission number 3.5.4.4) is an enzyme that catalyses the irreversible conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. This enzyme has been proposed in human beings as a biomarker of inflammation (Mishra et al., 1994). ADA activity is increased in saliva in human beings with squamous cell carcinoma of the tongue (Rai et al., 2011). In experimental infection of dogs with *Ehrlichia canis*, serum ADA activity decreased at 12 days, then increased at 30 days after infection (Da Silva et al., 2013). ADA activity is decreased in bitches with mammary tumours compared to unaffected dogs (Machado et al., 2015) and in dogs with leishmaniasis compared to uninfected dogs (Tonin et al., 2016). Therefore, the role of ADA as a marker of inflammation in dogs is still to be fully elucidated.

Dogs may perceive different degrees of stress when they face new situations, such as a visit to a veterinary clinic or being hospitalised. This induced stress reaction can involve the

* Corresponding author.
E-mail address: roman.dabrowski@up.lublin.pl (R. Dąbrowski).

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sympathetic-adrenomedullary (SAM) system and the hypothalamic-pituitary-adrenal (HPA) axis. Salivary chromogranin A and α-amylase (Enzyme Commission number 3.2.1.1) are considered to be markers of activation of the SAM system in the dog, whereas cortisol concentrations increase in dogs after activation of the HPA (Contreras-Aguilar et al., 2017a; Srithunyarat et al., 2017). Serum cortisol concentrations are increased in bitches with pyometra and decrease after treatment (Reinoldes, 2010).

The aims of this study were to evaluate salivary ADA as a possible indicator of inflammation in bitches with pyometra, as well as to evaluate salivary α-amylase as a marker of activation of the SAM system. To achieve these aims, a commercial assay for ADA was validated in canine serum and saliva, and salivary ADA and α-amylase were evaluated, in dogs with pyometra and unaffected dogs. Changes in these analytes after treatment were assessed to determine their possible usefulness for monitoring responses to treatment.

Materials and methods

Animals

Bitches undergoing ovariohysterectomy at the Department and Clinic of Animal Reproduction, University of Life Sciences, Lublin, Poland, from November 2016 to May 2017 were included in this study. The first group comprised unaffected bitches that were classified as healthy after complete physical examination, haematology and biochemistry. The second group comprised bitches diagnosed with pyometra after clinical examination and additional testing, including haematology and biochemistry. In most cases the clinical examination revealed pyrexia, polydipsia, polyuria, anorexia, apathy and abnormal colour of mucous membranes. Vaginal discharge was observed in all diseased animals and there were abundant degenerate neutrophils with intracellular bacteria on cytological examination of vaginal smears. Findings on abdominal ultrasound in all affected bitches were consistent with pyometra.

Bitches with pyometra were treated by ovariohysterectomy after premedication with 30 μg/kg medetomidine (Domitor, Pfizer) intramuscularly, 0.2 mg/kg butorphanol (Torbugesic, Zoetis) intravenously and 2 mg/kg ketamine (VetaKetam, VetAgro) intravenously. Anaesthesia was maintained with 2% isoflurane (Iso-Vet, Chanelle). Cephalexim (Cefalexim 18%, ScanVet) was administered for 5 days post-surgery at 10 mg/kg subcutaneously. Pyometra was confirmed by histopathological examination of the uterus and ovaries by veterinary pathologists in the Department of Pathological Anatomy, University of Life Sciences, Lublin, Poland. Uterine pus from all affected bitches was submitted for bacteriology. Bitches with pyometra were classified according to their physical status using a scoring system adapted from the American Society of Anaesthesiologists (ASA grade; Table 1) (Thurmon et al., 1996).

Collection of samples

Saliva was collected using Salivette tubes (Sarstedt) containing a sponge. Each dog was allowed to chew the sponge until it was thoroughly moist, then the sponges were placed in the tubes and kept refrigerated during transportation to the laboratory. At the laboratory, the tubes were centrifuged at 3000 *g* and 4 °C for 10 min. Blood samples were obtained by cephalic vein venepuncture using 21 G needles and 5 mL syringes.

A portion of each blood sample was transferred into tubes (Medlab Products) containing ethylenediaminetetraacetic acid (K_3-EDTA) for haematology. The remainder of each blood sample was drawn into Vakuette silicone test tubes (Greiner Labortechnik) and allowed to clot for 30 min, then the tubes were centrifuged (15 min) at 3000 *g* to obtain serum. Seven out of the 26 bitches with pyometra were sampled after treatment for both serum and saliva. In these animals, samples were collected before surgery (T0), at 3 days (T1) and at 10 days (T2) after surgery. The research protocols used in the current study were approved by the University of Lublin institutional animal care and ethics committee (approval number 27/2015, date of approval 18th May 2015).

Analytical measurements

Complete blood cell counts were performed with a haematology analyser (Scil Vet Plus+ Horiba ABX) and leukocyte differential counts were performed manually by examination of routinely stained blood smears. Routine biochemistry included testing for alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, urea and creatinine (MindrayBS-130 W; Shenzhen Mindray Bio-medical Electronics Company) These analyses were performed in the Department and Clinic of Animal Reproduction, Faculty of Veterinary Medicine, University of Life Sciences, Lublin, Poland.

Serum and saliva samples were frozen at −76 °C (Polar 110 H, Angelantoni Industries) until transported to the Interdisciplinary Laboratory of the University of Murcia, Spain (Interlab-UMU) for further analyses. Samples were shipped on dry ice and the time elapses between sampling and shipping was < 6 months. Serum C-reactive protein (CRP) concentrations were measured using a human immunoturbidimetric test (CRP OSR 6147 Olympus Life and Material Science) previously validated in dogs (Martínez-Subiela and Cerón, 2005). Serum haptoglobin (Hp) concentration was measured using a commercial colorimetric method (Tridelta Phase Range Haptoglobin Kit, Tridelta Development), previously validated in the authors' laboratory for canine samples (Martínez-Subiela and Cerón, 2005). These analytes were measured using a chemistry analyser (Olympus AU2700, Olympus Diagnostica). Serum cortisol concentrations were determined using a competitive chemiluminescence immunoassay (Immulite, Diagnostic Products Corporation) previously validated for use in dogs (Singh et al., 1997).

Total ADA activity was measured in serum and saliva samples using a spectrophotometric automated method (Adenosine Deaminase Assay Kit, Diazyme Laboratories) validated at Interlab-UMU (see Appendix: Supplementary material). Salivary α-amylase activity was measured using a commercially available method (α-Amylase, Beckman Coulter) previously validated for canine saliva at Interlab-UMU (Contreras-Aguilar et al., 2017b). Total protein concentrations in saliva samples were determined using a colorimetric method (Protein in Urine and CSF, Spinreact) to evaluate the influence of saliva concentration on the results. ADA and α-amylase activities are expressed as IU/g of protein. These analytes were measured in an automated clinical chemistry analyser (Olympus AU2700).

Statistical analysis

Means, standard deviations, coefficients of variation (CVs) and regression analyses were determined. Data obtained from healthy and diseased animals were evaluated for normality of distribution, using Shapiro–Wilk/Kolmogorov–Smirnov tests. Multivariate linear regression was performed to assess whether age could be a significant predictor for the analytical parameters. To assess any difference between groups, normally distributed data were checked using a two-tailed *t* test. The Mann–Whitney U was used for data that was not normally distributed. For the seven bitches in which follow-up was recorded, changes were assessed with a one-way analysis of variance of repeated measures, followed by Tukey's post-hoc test for normal data, and Friedman's test followed by Dunn's multiple comparisons test in the case of non-normally distributed data. Effect size was assessed by Cohen's *d* and *f* coefficients: $d > 0.8$ and $f > 0.4$ were considered to be large size effects (Cohen, 1988). Correlations of salivary ADA and α-amylase activities with ASA scores, inflammatory markers (WBC and band counts, CRP, Hp and rectal temperature) and stress biomarkers (serum cortisol) were studied by Spearman correlation assays and linear regression plots were constructed. Data analyses were performed using Excel 2000 (Microsoft Corporation), GraphPad Prism version 5 for Windows (GraphPad Software) and SPSS Statistics version 24 (IBM Corporation). Values of $P < 0.05$ were considered to be statistically significant.

Results

Clinical findings

The healthy group included 18 bitches 0.5–6 years of age (mean 2.8 years), body weight 5.0–42 kg (mean 24.2 kg), body condition score (BCS) 2.0–4.0 (mean 3.2 out of 5) and rectal temperature

Table 1
Classification of physical status using a modification of the American Society of Anaesthesiologists (ASA) grade (Thurmon et al., 1996).

Category	Physical status	Examples of conditions in this category
I	Normal healthy animal	No discernible disease; animals entered for ovariohysterectomy or castration
II	Animal with mild systemic disease	Skin tumour; fracture without shock; uncomplicated hernia; cryptorchidectomy; localised infection; compensated cardiac disease
III	Animal with severe systemic disease	Pyrexia; dehydration; anaemia; cachexia; moderate hypovolaemia
IV	Animal with severe systemic disease that is a constant thread to life	Uraemia; toxaemia; severe dehydration and hypovolaemia; anaemia; cardiac decompensation; emaciation; high fever
V	Moribund animal not expected to survive 1 day with or without operation	Extreme shock and dehydration; terminal malignancy or infection; severe trauma