Adenosine deaminase (ADA) characterization in saliva and serum samples of different animal species

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Abstract

Background The enzymatic activity of total adenosine deaminase (tADA) and its isoenzymes ADA1 and ADA2 were studied in canine, equine, porcine and bovine serum and saliva. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was used as a specific ADA1 inhibitor at different concentrations, and the distribution of the isoenzymes in serum and saliva was studied in different inflammatory situations in each species.

Results Total ADA was present in serum and saliva of healthy animals of the four species. EHNA concentration of 0.47mM was needed for ADA1 inhibition in canine and porcine samples (serum and saliva) and bovine saliva, whereas for equine saliva 0.94mM was needed. ADA2 activity was not detected in bovine serum and was very low or absent in equine serum and bovine saliva. An automated procedure to measure ADA2 consisting of adding EHNA to a commercial reagent for tADA measurement provided repetitive (coefficients of variation <8.8% in serum and <10% in saliva) and accurate (linearity under dilution approaches gave R² >0.90) results, being equivalent to a manual incubation of the sample with EHNA at a similar concentration. Salivary tADA, as well as ADA1 and ADA2, were higher in dogs with leishmaniosis, horses with acute abdominal disease and pigs with lameness than in healthy animals. ADA showed significant correlation with serum ferritin in dogs (r = 0.602, P < 0.01; r = 0.555, P < 0.05; and r = 0.632, P < 0.01; respectively for tADA, ADA1 and ADA2) and serum C-reactive protein in pigs (r = 0.700, P < 0.01, for both tADA and ADA1; r = 0.770, P < 0.001, for ADA2), whereas salivary ADA2 significantly correlated with serum amyloid A in the horse (r = 0.649, P < 0.01). In the cow, salivary tADA and ADA1 significantly increased after calving, being correlated with total white blood cell count (r = 0.487, P < 0.05, both).

Conclusions ADA activity, as well as its isoenzymes, can be measured in serum and saliva of different animal species, and the salivary levels of these analytes could potentially be inflammatory biomarkers in these species.

Background

Adenosine deaminase (ADA, EC number 3.5.4.4) is an enzyme that can be found in several tissues and fluids and it has two different isoenzymes. The isoenzyme ADA1 is mainly present in lymphoid tissue and plays a role in the differentiation of B and T lymphocytes, as well as in maturation from monocyte to macrophage [1, 2]. ADA2 is the predominant in human plasma [3] but it has a lower affinity to adenosine compared with ADA1 [4, 5, 6]. Although its function is poorly understood, ADA2 is probably involved in the haematopoietic system [7, 8], being secreted by premonocytic cells as a growth factor for the monocyte lineage [4, 5, 6].

Total ADA (tADA) activity can be increased in serum in those diseases in which the number of T lymphocytes increases [9, 10] and due to this fact it has been used as a marker of cell-mediated immunity [11, 12] and inflammation [13]. Several diseases have been reported to increase serum ADA activity in humans, including inflammation such as chronic tonsillitis, rhinosinusitis or otitis media [3],
immunomediated disorders such as systemic lupus erythematosus [14, 15] or rheumatoid arthritis [16], and malignancies such as chronic lymphocytic leukemia [17], breast cancer [18] or bladder cancer [19]. Serum ADA has been also measured in veterinary species being higher in inflammation in dogs [20] and cows [21]. In contrast, decreased tADA activity in serum has been found in pigs with lameness [22].

Saliva is an organic fluid with potential usefulness for biomarkers determination, not only for oral diseases but also for systemic pathologies [23]. It can be easily and safely collected, even by untrained personnel, causing a minimum disturbance to the animal [24]. In humans, ADA activity was successfully measured in saliva being increased in local pathologies such as oral malignancies [25] or Sjögren’s syndrome [26], and systemic pathologies such as obesity [27]. In animals, increased tADA activity in saliva has been described in bitches with pyometra [28] and pigs with lameness [22], probably due to the presence of a systemic inflammatory process. Recently, tADA has been also measured in saliva from sheep, cow and horses [29, 30, 31].

Regarding ADA isoenzymes, the ADA2 activity is 100-fold lower than ADA1 in human serum [7], while in the serum of cows, ADA2 isoenzyme has not been detected [21, 32]. However, to the best of authors’ knowledge, ADA activity has not been characterized in the serum of other animal species. In saliva, there are no studies about the ADA isoenzyme activity characterization and distribution in any animal species with the exception of the pig [33].

The aim of this report was to study the enzymatic activity of tADA and its two isoenzymes ADA1 and ADA2 in serum and saliva samples of four different animal species (dog, horse, pig and cow) in order to characterize this enzyme and its isoenzyme distribution. For the determination of the isoenzymes activity of ADA an automated assay, in which the specific ADA1 inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was added to the reaction mixture in order to isolate ADA2 activity, was validated. In addition, changes of the tADA and its isoenzymes were studied in different inflammatory situations and the correlation of these analytes with traditional inflammatory markers such as acute-phase proteins or total white blood cell count (WBC) was evaluated.

**Methods**

**Sampling**

In all cases, saliva was obtained previous to blood in order to avoid any possible influence of stress associated with blood collection on the saliva results. Saliva was collected using Salivette tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nürnberg, Germany) containing a sponge (Esponja Marina, La Griega E. Koronis, Madrid, Spain) instead of a cotton swab. The animals were allowed to chew the sponge until thoroughly moist with the help of a flexible thin metal rod. Then, the sponge was placed into the Salivette tube. Venous blood was obtained from venipuncture of the jugular (dogs, horses and pigs) or caudal (cows) veins, using tubes without additive (BD Vacutainer, Franklin Lakes, NJ, USA) and allowed to clot. All samples were kept in ice until arrival at the laboratory for processing (less than 2 hours).
Once at the laboratory, all saliva samples were visually checked and no reddish samples indicating blood contamination were included in the study. The saliva samples were centrifuged (Universal 320R, Hettichzentrifugen, Tuttingen, Germany) at 3000 x g and 4° C for 10 min. Then, the supernatant was collected in plastic tubes of 1.5mL (Eppendorf), discarding the sediment. Blood tubes were centrifuged in similar conditions than saliva, and serum was collected in Eppendorf. Saliva and serum specimens were stored at -80°C until analysis.

ADA assay

ADA was analyzed with a commercially available spectrophotometric automated assay (Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA). The fundament of this method is as follows: the substrate adenosine is deaminated to inosine by ADA. Inosine is then converted to hypoxanthine by purine nucleoside phosphorylase, which is then converted to uric acid and hydrogen peroxide by xanthine oxidase. The amount of peroxidase produced in the reaction is proportional to the ADA activity in the sample, and it is quantified by reaction with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline and 4-aminoantipyrene in the presence of peroxidase, leading to a quinine dye kinetically monitored at a 550 nm wavelength [34]. This method was adapted to an automated analyzer (Olympus AU400, Olympus Diagnostica GmbH, Ennis, Ireland) following the manufacturer's protocol with some modifications for its use in saliva. The lower limit of detection (LLLOD) of this method was 0.07 IU/L, following previously published data [28].

For isoenzyme determinations, the specific ADA1 inhibitor EHNA (Merck KGaA, Darmstadt, Germany) was used. At a proper concentration, EHNA inhibits ADA1 isoenzyme whereas ADA2 remains unaffected [35]. Therefore, tADA and ADA2 isoenzyme can be determined when samples are analyzed in the absence and presence of EHNA, respectively, and the isoenzyme ADA1 is calculated from the difference between both measurements.

Optimization of EHNA concentration for ADA2 measurement in serum and saliva in different species

In order to determine the appropriate concentration of EHNA that should be used in each species for total inhibition of ADA1 isoenzyme, the following samples were used:

**Dogs:** Samples were obtained from five healthy Beagle dogs (*Canis lupus familiaris*). All dogs were neutered males, 3.5 ± 0.8 years old and 26.0 ± 7.1 Kg body weight. The animals were located in the Experimental Farm of the University of Murcia (Murcia, Spain).

**Horses:** Samples were obtained from five healthy horses (*Equus caballus*), one stallion and four geldings, mean age 10.0 ± 5.1 years old, with body condition score (BCS) 3.4 ± 0.5, including three Spanish horses, one Spanish Arabian and one Warmblood. Horses showed no clinical signs of pain or discomfort after a physical examination.

**Pigs:** Samples were collected from five apparently healthy growing pigs (*Sus scrofa domesticus*), Large White x Large White males with 2-3 months-old in the last phase of fattening, housed in the Experimental
Farm of the University of Murcia (Murcia, Spain).

**Cows:** Samples were obtained from five Holstein dairy cows (*Bos taurus*), lactation 3.5 ± 1.0, mean age 5.3 ± 1.4 years old, days in milk 234.8 ± 9.4, from a commercial dairy herd located in the southeast of Spain. The animals were healthy at physical examination.

Each sample was separated in aliquots. Then, EHNA was added to the saliva and serum samples at increasing concentrations (0.1, 1.0, 4.0 and 8.0 mM), whereas an equal volume of diluent was added to one aliquot that was used as control. The proper concentration of EHNA in each species was selected based on its ability to give the same result in ADA value with this concentration than when a higher EHNA concentration was used since this would indicate that at this concentration there is a total inhibition of ADA1.

### Development and validation of an automated assay for ADA2 isoenzyme measurement

An automated assay for the measurement of the ADA2 isoenzyme was developed in which EHNA was added to the reagent 1 at an adequate concentration for each species based on the results of the tests described in the previous point. In each species a similar volume of samples obtained from 10 different animals (five with low and five with high ADA2 activity) were mixed in order to prepare two pools of serum and two pools of saliva with different ADA2 activity. Inter-assay imprecision and linearity under dilution were evaluated in serum and saliva samples from the different species by calculating the intra-assay coefficient of variation (CV) and linear regression coefficient, following previously published protocols [22, 28].

In addition, as a part of the validation, the results obtained with the automated procedure were compared with those obtained after the manual addition of the inhibitor. For this approach, the pig was selected as a model because of its high activity in saliva samples. For this purpose, serum and saliva samples with low (N = 15) and high (N = 18) ADA2 activity were obtained. ADA2 isoenzyme was analyzed manually by adding EHNA to the samples and automatically by adding EHNA to the reagent 1, in such concentrations that in both procedures the same final concentration of EHNA in the reaction mixture was achieved.

### Clinical validation

For this purpose, tADA and its isoenzymes were measured by using the fully automated method in the following samples:

**Dog:** Samples from 20 dogs were included and divided into two groups. The healthy dog group were integrated by samples of 10 client-owned dogs belonging to the staff of the Animal Medicine and Surgery Department of the University of Murcia. They were 3.9 ± 1.5 years old, with BCS 4.0 ± 1.0, and included three Retrievers, three mixed breed dogs and one of the following breeds: Beagle, French bulldog, Scottish terrier and Brie shepherd. All were neutered males apparently healthy after physical and haematological examinations. The other 10 samples were from client-owned dogs arriving at the Veterinary Teaching Hospital of the University of Murcia, naturally infected with *Leishmania infantum* with clinical signs. The
group with leishmaniosis included three mixed breed dogs and one of the following breeds: Retriever, French bulldog, Collie, Beagle, Irish setter, German shepherd and Rottweiler. There were five males and five females, with 3.0 ± 1.0 years-old, and BCS 2.7 ± 0.5. The clinical signs described in the 10 dogs with leishmaniosis included lymphadenopathy and anaemia (1/10), skin lesions and uveitis (2/10), weight loss and hypoalbuminaemia (3/10), and hyperglobulinaemia (6/10). The diagnoses were based on positive polymerase chain reaction (PCR) and serology results. The concentrations of the acute phase protein ferritin in serum, a biomarker of systemic inflammation in this disease, was analyzed as previously described [36], ensuring that all healthy individuals had values <190µg/L.

**Horse:** Samples from 20 horses (10 considered as healthy after physical and blood examinations, and 10 with acute abdominal pain) were included. The healthy animals were male horses admitted for castration or routine health check and included different breeds (seven Spanish horses, one Spanish-Arabian, one Warmblood and one crossbreed), mean age 8.0 ± 4.2 years-old, and BCS 3.5 ± 0.4. They showed no clinical signs of abdominal pain or other diseases during physical examination, as well as haematological or biochemical abnormalities. The group of diseased animals were integrated by horses with acute abdominal disease. This group included animals with different breeds (five Spanish horses, two Warmblood horses, one Lusitanian horse, one Holsteiner and one crossbreed) all males, mean age 11.3 ± 3.3 years-old, and BCS 3.4 ± 0.7. The diagnoses were based on clinical history, physical examination, haematology and plasma biochemistry, transabdominal ultrasonography, rectal examination, nasogastric intubation and on laparotomy findings in surgical cases. The following diagnoses were obtained: three colon impaction with large colon displacement, three stomach impaction, one nephrosplenic entrapment, one impaction of the pelvic flexure, one large colon displacement, and one enteritis. Serum levels of the acute phase protein serum amyloid A (SAA) were measured as a marker of acute systemic inflammation as previously described [37]. All healthy animals showed SAA values <2.3µg/mL.

**Pig:** Samples from 20 animals (Large White x Large White males with 2-3 months-old in the last phase of fattening) housed in the Experimental Farm of the University of Murcia (Spain) were used. The healthy group was composed of 10 apparently healthy pigs after physical examination at the farm. The diseased group was composed of 10 lame pigs. The presence of lameness was considered based on the observation of the animals according to the scoring system published by Main et al. [38]. An animal was considered lame when it achieved a score ≥ 1 in the lameness score. Serum C-reactive protein (CRP) concentration was used as a marker of systemic inflammation, and healthy animals gave values <20µg/mL, as previously described [33].

**Cow:** Samples from 10 dairy cows (seven Holstein, two Montbellier and one crossbreed), mean age 4.9 ± 1.6 years-old, parity 3.4 ± 1.6 and BCS 3.4 ± 0.7, from a commercial dairy herd located in the southeast of Spain were used. All animals were at the last phase of gestation, apparently healthy and no lameness, mastitis, metritis, ketosis or other health issues were observed. Blood and saliva samples were obtained 13 ± 7 days before calving (Before calving) and at the day of calving (At calving), between January and February of 2019, to avoid any change in the results due to seasonal reasons. The acute phase protein
haptoglobin (Hp) [31] and the total WBC (Advia 120 haematology Analyzer, Siemens Healthcare GmbH, Erlangen, Germany) were used as indicators of inflammation.

Statistical analysis

Data obtained from ADA measurements were analyzed for normality, giving a non-normal distribution. The changes due to the presence of EHNA at different concentrations were assessed by Friedman's followed by Dunn's multiple comparison tests. The concentration that provided statistically significant results with the previous ones but from which there were no more changes was considered as the most appropriate for completely inhibit ADA1 activity. ADA2 results obtained after manual and automated inhibition in the 33 samples (15 with low and 18 with high ADA2 activity) of porcine serum and saliva were compared by linear regression and Bland-Altman plot in which difference between methods were plotted against the average value. Unpaired Mann-Whitney test was used to compare tADA and isoenzymes results between healthy and diseased animals, and Wilcoxon signed rank test was used to compare tADA and isoenzymes results between the two different measurements performed in cows. Spearman correlation coefficients (r) were calculated between ADA results and the biomarkers of inflammation. The correlations were considered according to the r value as very high (≥ 0.90), high (0.70-0.89), moderate (0.50-0.69), low (0.30-0.49), and negligible (< 0.30), following the Rule of Thumb [39]. Data analyses were performed using Excel 2000 (Microsoft Corporation, Redmond, WA, USA) and Graph Pad Software Inc (GraphPad Prism, version 5 for Windows, Graph Pad Software Inc, San Diego, CA, USA). A P value less than 0.05 was considered as significant.

Results

Values of total ADA and distribution of its isoenzymes in serum and saliva in healthy individuals from different species

Results obtained in dog samples are shown in Fig. 1A. Before incubation with EHNA, serum and saliva samples showed similar ADA activities. In both serum and saliva samples, significant inhibition was achieved with 1 mM EHNA, but complete inhibition was achieved with 4 mM in both saliva and serum. When 4 mM EHNA was used, median (25th -75th percentiles) for ADA1 and ADA2 in serum was 6.32 (5.66–9.91) IU/L and 1.28 (1.21–1.50) IU/L, respectively. In saliva, those values were 5.77 (3.76–6.58) IU/L and 2.20 (1.50–3.24) IU/L for ADA1 and ADA2, respectively.

Results obtained in horse samples are shown in Fig. 1B. In serum, tADA activity was very low before adding EHNA, with 1/5 samples having values under the LLOD. Since no change was observed between 1 and 4 mM EHNA and most values were under the LLOD of the method, a higher EHNA concentration was not tested. In contrast, saliva samples showed higher ADA activities than the LLOD of the method in all the samples. The statistical analysis showed that ADA was significantly inhibited with 4 mM EHNA, although a concentration of 8 mM would be most appropriate for complete ADA1 inhibition. At 4 mM EHNA, the median (25th -75th percentiles) for ADA1 and ADA2 in serum was 0.12 (0.06–0.14) IU/L and
0.07 (0.00-0.21) IU/L respectively. In saliva, those values at 8 mM EHNA concentration were 18.70 (18.29–39.34) IU/L and 0.98 (0.61–5.47) IU/L for ADA1 and ADA2, respectively.

Results obtained with pig samples are shown in Fig. 1C. Saliva had a much higher ADA activity than serum. In serum, significant inhibition was observed with 4 mM EHNA, with no further reduction in activity with a higher concentration. Similar results were observed in saliva. All results were above the LLOD of the method. At 4 mM EHNA, median (25th -75th percentiles) for ADA1 and ADA2 in serum were 7.67 (4.74–8.30) IU/L and 1.67 (1.00-1.91) IU/L, respectively. In saliva, those values were 782.20 (496.65-1078.27) IU/L and 1.53 (1.30–2.35) IU/L for ADA1 and ADA2, respectively.

Results obtained in cow samples are shown in Fig. 1D. Before incubation with EHNA, serum and saliva samples provided similar tADA results. In serum, inhibition was considered significant with respect to initial values at 1 mM EHNA, with 4/5 values under the LLOD; no more changes were observed with a higher concentration. In saliva, 4 mM EHNA was required to achieve a significant inhibition, with no samples below the LLOD. Since practically no changes were observed between 1 and 4 mM EHNA in both serum and saliva samples, a higher EHNA concentration was not tested. At 4 mM EHNA, median (25th -75th percentiles) for ADA1 in serum was 4.20 (3.87–7.56) IU/L, whereas ADA2 was negligible. In saliva, those values were 13.55 (6.13–20.96) IU/L for ADA1, and 0.29 (0.13–0.44) IU/L for ADA2.

Validation of the automated assay for ADA2 measurement

Intra-assay CV was lower than 8.8% in serum and 10% in saliva (Table 1). Linearity under dilution approach showed $R^2 > 0.90$ in all cases (Table 2). LLOD was set at 0.07 UI/L. The automated ADA2 determination was not validated in serum from horses and cows, since values were under the LLOD of the assays.
Table 1
Intra-assay precision results. They were obtained from two pools (with high and low adenosine deaminase 2 activity) of serum and saliva from dogs, horses and pigs. Mean ± standard deviation (coefficient of variation) from 5 replicates of each pool of samples and species are indicated. Mean and standard deviations are expressed in IU/L, coefficients of variation in %.

|       | Serum          | Saliva         |
|-------|----------------|----------------|
|       | Mean ± standard deviation (coefficient of variation) |       |
| Dog   |                |                |
| High  | 1.79 ± 0.10 (5.63) | 3.83 ± 0.13 (3.43) |
| Low   | 0.62 ± 0.03 (4.60) | 0.87 ± 0.09 (9.96) |
| Horse |                |                |
| High  | -              | 4.77 ± 0.14 (2.89) |
| Low   | -              | 0.66 ± 0.06 (8.60) |
| Pig   |                |                |
| High  | 4.30 ± 0.18 (4.15) | 6.06 ± 0.06 (0.97) |
| Low   | 0.91 ± 0.08 (8.72) | 0.67 ± 0.04 (5.41) |
| Cow   |                |                |
| High  | -              | 1.99 ± 0.11 (5.75) |
| Low   | -              | 0.24 ± 0.02 (6.59) |
Table 2
Linearity under dilution for the automated measurement of adenosine deaminase isoenzyme 2 (ADA2). Results were obtained from two pools of serum and two pools of saliva with high and low enzymatic activities.

| Species | Sample | ADA2 (IU/L) | Slope       | Y-intercept | R²  | P       |
|---------|--------|-------------|-------------|-------------|-----|---------|
| Dog     | Saliva | 3.90        | 1.14 ± 0.07 | -0.04 ± 0.08| 0.98| < 0.001 |
|         |        | 0.64        | 1.35 ± 0.15 | -0.02 ± 0.03| 0.95| < 0.001 |
|         | Serum  | 1.79        | 1.14 ± 0.10 | -0.01 ± 0.05| 0.95| < 0.001 |
|         |        | 0.98        | 1.16 ± 0.14 | -0.01 ± 0.05| 0.94| < 0.001 |
| Horse   | Saliva | 4.77        | 1.00 ± 0.12 | -0.15 ± 0.17| 0.91| < 0.001 |
|         |        | 0.66        | 1.02 ± 0.05 | 0.05 ± 0.04 | 0.99| < 0.001 |
| Pig     | Saliva | 6.06        | 1.02 ± 0.03 | 0.00 ± 0.05 | 0.99| < 0.001 |
|         |        | 0.67        | 1.04 ± 0.13 | -0.04 ± 0.04| 0.92| < 0.001 |
|         | Serum  | 4.30        | 1.38 ± 0.06 | -0.35 ± 0.07| 0.99| < 0.001 |
|         |        | 0.91        | 1.34 ± 0.13 | -0.21 ± 0.06| 0.95| < 0.001 |
| Cow     | Saliva | 2.20        | 1.65 ± 0.14 | 0.06 ± 0.09 | 0.96| < 0.001 |
|         |        | 0.56        | 1.41 ± 0.21 | -0.05 ± 0.04| 0.92| < 0.01  |

R²: Coefficient of linear regression; P: P value.

For the comparison between manual and automated ADA2 determination protocols in porcine samples, a final EHNA concentration in the reaction mixture of 0.47 mM (equivalent with a sample incubation at 4 mM) was selected since the previous results showed that this concentration was able to completely inhibit ADA1 isoenzyme in both serum and saliva samples. Linear regression between ADA2 results obtained with the manual incubation and the automated procedure showed slope significantly close to 1 and Y-intercept significantly close to zero, with a R² > 0.94 (P < 0.001) for serum and R² > 0.99 (P < 0.001) for saliva (Fig. 2). Bland-Altman plots showed bias of 0.21 ± 0.40 IU/L for serum and 0.29 ± 0.30 IU/L for saliva (Fig. 3).

Clinical validation

Results of the different experiments performed for the clinical validation are shown in Table 3. No statistically significant differences were observed between healthy dogs and dogs with leishmaniasis in serum tADA or its isoenzymes. However, salivary tADA, as well as both isoenzymes ADA1 and ADA2, were statistically higher in the dogs with leishmaniasis, showing moderate correlation with serum ferritin (r = 0.602, P < 0.01, for tADA; r = 0.555, P < 0.05, for ADA1; and r = 0.632, P < 0.01, for ADA2).
Table 3
Adenosine deaminase activity in animals with and without inflammatory conditions. Total adenosine deaminase (tADA), and isoenzymes ADA1 and ADA2, obtained in healthy animals and dogs with clinical leishmaniosis, horses with acute abdominal disease, pigs with lameness and healthy cows before and after calving. Results are expressed in median (25th -75th percentiles) IU/L.

|            | Serum                          | Saliva                          |
|------------|--------------------------------|---------------------------------|
|            | Dogs                           | Canine leishmaniosis            | Healthy                          | Canine leishmaniosis            |
|            | N = 10                         | N = 10                          | N = 10                           | N = 10                           |
| tADA       | 12.90 (8.60–16.83)             | 9.05 (7.85–16.25)               | 4.25 (2.75–5.65)                 | 10.60* (3.55–12.18)              |
| ADA1       | 12.12 (7.77–15.60)             | 8.78 (7.24–14.70)               | 2.81 (1.78–4.54)                 | 7.49* (2.07–10.15)               |
| ADA2       | 1.00 (0.69–1.25)               | 0.61 (0.42–1.19)                | 1.16 (0.87–1.61)                 | 2.11* (1.08–3.00)                |
|            | Horses                         | Healthy                         | Acute abdominal disease          | Healthy                          | Acute abdominal disease          |
|            | N = 10                         | N = 10                          | N = 10                           | N = 10                           |
| tADA       | 0.23 (0.16–0.35)               | 0.43 (0.01–0.90)                | 46.30 (18.74–61.48)             | 162.20* (47.00–379.30)           |
| ADA1       | 0.15 (0.05–0.20)               | 0.28 (0.01–0.52)                | 43.84 (18.26–60.80)             | 158.60* (42.36–368.80)           |
| ADA2       | 0.06‡ (0.00–0.19)              | 0.01‡ (0.00–0.51)               | 0.90 (0.26–2.50)                 | 5.35** (2.02–10.53)              |
|            | Pigs                           | Healthy                         | Lameness                         | Healthy                          | Lameness                         |
|            | N = 10                         | N = 10                          | N = 10                           | N = 10                           |
| tADA       | 10.42 (8.34–13.70)             | 9.61 (6.86–10.47)               | 495.10 (347.00–823.40)           | 1829.00** (814.50–2683.00)       |
| ADA1       | 8.87 (7.11–12.53)              | 6.29* (4.07–7.91)               | 493.00 (346.40–822.40)           | 1823.00** (811.60–2677.00)       |
| ADA2       | 1.40 (0.79–1.91)               | 2.31* (1.45–4.36)               | 1.12 (0.55–2.06)                 | 4.33*** (2.36–8.61)              |

‡: under the lower limit of detection of the assay; *: P < 0.05; **: P < 0.01; ***: P < 0.001.
| Serum | Saliva |
|-------|-------|
| **Cows** | Before calving | At calving | Before calving | At calving |
|       | N = 10 | N = 10 | N = 10 | N = 10 |
| **tADA** | 7.14 (6.01–8.21) | 8.79* (6.93–13.47) | 5.51 (2.93–6.96) | 22.40** (13.19–28.61) |
| **ADA1** | 7.14 (6.01–8.21) | 8.79* (6.93–13.47) | 5.20 (2.55–6.57) | 22.30** (12.63–28.40) |
| **ADA2** | 0.00‡ (0.00–0.00) | 0.00‡ (0.00–0.00) | 0.36 (0.13–0.46) | 0.14 (0.02–0.29) |

‡: under the lower limit of detection of the assay; *: P < 0.05; **: P < 0.01; ***: P < 0.001.

In horses, serum provided tADA activity values under the LLOD in 2/10 healthy animals and 3/10 with colic, with no statistical differences between groups. ADA2 in serum was under the LLOD in all samples. In contrast, all values found in saliva were over the calculated LLOD of the method, with values of tADA and both isoenzymes being statistically higher in horses with colic. ADA2 in saliva moderately correlated with serum SAA (r = 0.649, P < 0.01).

In pigs, serum ADA1 were statistically lower in the diseased group than in the healthy group, whereas serum ADA2 was statistically higher. ADA1 in serum showed a moderate negative correlation with serum CRP (r = -0.511, P < 0.05). In saliva, tADA and both isoenzymes were statistically higher in the diseased group. Salivary tADA, ADA1 and ADA2 showed a significant high correlation with serum CRP (r = 0.700, P < 0.01, for both tADA and ADA1; r = 0.770, P < 0.001, for ADA2).

In cows, tADA in serum showed a significant increase at calving, being entirely due to ADA1, since ADA2 isoenzyme provided negligible results in all cases. In saliva, tADA activity showed a 4-fold increase at calving, as well as ADA1 isoenzyme. No significant changes were observed in the isoenzyme ADA2. Salivary tADA and ADA1 showed a low positive correlation with WBC count (r = 0.487, P < 0.05), whereas no correlation was observed with Hp.

**Discussion**

EHNA is a specific ADA1 isoenzyme inhibitor that, at a proper concentration, is able to completely inhibit ADA1 isoenzyme whereas remains ADA2 unaffected [35]. In our report, the adequate EHNA concentration was selected for each species based on having two criteria: (1) the concentration was able to decrease tADA activity, (2) a higher concentration did not provide any significant reduction in the enzymatic activity. The results indicated that an EHNA concentration of 0.12 mM in the reaction mixture (equivalent to incubate samples at 1 mM) would be enough to completely inhibit ADA1 isoenzyme in cow serum.
However, in canine and porcine samples (both serum and saliva), as well as in bovine saliva, a higher concentration of at least 0.47 mM in the reaction mixture (4 mM in the sample) would be needed. Whereas a concentration of 0.94 mM in the reaction mixture (8 mM in the sample) would be more appropriate in equine saliva, since produced an additional significant inhibition than 0.47 mM, and in this species, no additional reduction on activity was observed with a concentration of 1.18 mM in the reaction mixture (data not shown). These concentrations are higher than that recommended in the bibliography for humans of 0.1 mM in the reaction mixture [35]. Altuğ et al. [21] used an EHNA concentration of 0.2 mM in the reaction mixture for measuring ADA2 in serum from cows, which is also higher than the concentration recommended for humans, although our results would indicate that 0.12 mM would be enough. Therefore, the sensitivity to the inhibitor could depend on the animal species and the sample, being bovine serum the most sensitive and equine saliva the less.

An automated procedure of quantification of ADA2 was developed in our study. For this purpose, EHNA was added directly to a commercially available reagent designed to measure tADA in order to achieve a final concentration in the reaction mixture adequate for the species to be tested. This assay gave adequate values for imprecision and accuracy in all the species tested and could be used together with the previously validated tADA assay for the measurement of the different isoenzymes in the species of our study. For this purpose, a three-step procedure is proposed: (1) the sample is measured for evaluation of tADA with the commercial reagent by a previously validated assay [22, 28, 30]; (2) the sample is measured again with the commercial reagent adding EHNA at appropriate concentration for ADA2 estimation; and (3) the isoenzyme ADA1 is calculated by the difference between measurements of step 1 and 2. This procedure can be fully automated, would avoid pipetting errors and allows achieving results faster than with a manual inhibition of the samples.

In dogs, our results indicated that both canine serum and saliva had a similar tADA activity, being ADA1 the main contributor. When our assay was applied in dogs with leishmaniosis, no changes were detected in serum ADA with respect to healthy individuals. In contrast tADA, as well as its isoenzymes, were higher in saliva of dogs with canine leishmaniosis, correlating significantly with serum ferritin. This would indicate that ADA in saliva can increase in inflammatory conditions in dogs, in line with a previous report made in bitches with pyometra, which presented higher salivary tADA values than healthy dogs [28].

In our report, tADA value in horse serum was low but over the LLOD of the assay, or absent. The lack of ADA activity in serum of horses has been previously reported [40]. Over a 14-fold lower ADA activity has been found in horse lymphocytes when compared with humans [41], which could influence the very low ADA activity in horse serum. In contrast, horse saliva provided an abundant ADA activity, being ADA1 the main contributor. Further studies should be performed in order to found the source of this activity in saliva taking into account the low ADA activity that is present in serum and lymphocytes from this species. Higher values of salivary tADA and isoenzymes were detected in horses with the acute abdominal disease compared to healthy ones, especially in the case of ADA2, which showed a significant positive correlation with SAA and therefore could be considered as an inflammatory biomarker in horses.
Pigs showed the highest values of ADA, especially in saliva where tADA was over 100-fold higher than in serum, in agreement with previous reports [22]. The reason for this high ADA activity in porcine saliva is unknown and should be further studied. In our report, ADA1 was the main contributor to the tADA activity in both serum and saliva, in agreement with a recent report [29] but in contrast with a previous one that found ADA2 as the predominant form in porcine saliva [42]. In saliva, significant increases were found in tADA and ADA1 in pigs with lameness compared with healthy pigs but these changes were not detected in serum. In serum, only significant increases of ADA2 were found that were of lower magnitude than those found in saliva. Therefore, saliva should be the sample of election for detecting increases of tADA and isoenzymes in pigs with lameness. The high correlation that showed salivary tADA and isoenzymes with serum CRP would indicate that salivary ADA activities could be inflammatory biomarkers in this species.

In cows, we did not find ADA2 activity in serum, being these results in line with other reports [21, 32]. In saliva, tADA activity was of similar magnitude as in serum, and saliva from some healthy individuals did present ADA2 activity, although in low amounts. In our study, calving significantly increased ADA1 isoenzyme in both serum and saliva. An increase of ADA in serum after calving had been previously described [43], and therefore ADA has been proposed to be a marker of a proinflammatory status of the animal due to calving [44]. To the authors’ knowledge, this is the first report in which salivary ADA has been also reported to increase after calving in cows, being this change of higher magnitude of that reported in serum. The correlation observed between salivary ADA and the WBC count would indicate that also salivary ADA could act as a biomarker of inflammation in this species. The lack of correlation with serum Hp could be due to the slow response of this acute-phase protein. Further studies should be made in pathologic conditions to corroborate these findings.

This study presents as a main limitation that the number of animals used for the clinical validation was relatively low and that only one inflammatory situation was studied in each species. Therefore, it should be considered as a pilot one and it would be desirable to carry out studies in the future involving larger number of animals. In addition, different diseases or situations with associated inflammation should be also studied, to a better knowledge about how total ADA and their isoenzymes behave and their possible use as biomarkers.

**Conclusions**

Total ADA activity, as well as its different isoenzymes, could be measured in both serum and saliva in dogs, horses, pigs and cows by a simple and fast automated procedure described in this report. It should be taken into account that in this procedure the concentration EHNA which is used to inhibit ADA1 should be properly adjusted to the animal species in which it is going to be applied. When this procedure has been applied, differences in the distribution and activities of the isoenzymes in serum and saliva between the animal species have been observed. Overall, the results observed in this study would also indicate that ADA activity in saliva, due to its positive correlation with other inflammatory biomarkers, could potentially be an inflammatory biomarker in all the different species tested, although further studies
involving more animals and other pathologic conditions should be performed to corroborate these results.

**Abbreviations**

ADA
adenosine deaminase
tADA
total adenosine deaminase
ADA1
adenosine deaminase isoenzyme 1
ADA2
adenosine deaminase isoenzyme 2
BCS
body condition score
CRP
C-reactive protein
CV
coefficient of variation
EHNA
erythro-9-(2-hydroxy-3-nonyl) adenine
Hp
haptoglobin
LLOD
lower limit of detection
SAA
serum amyloid A
SD
standard deviation
WBC
white blood cell count

**Declarations**

Ethics approval and consent to participate

The experimental protocol was approved by the Bioethical Commission of Murcia University (CEEA 288/2017 and 235/2018) and followed the law RD 53/2013 regarding animal experimentation in Spain and with the European Directive 2010/63/EU concerning the protection of animals used for scientific purposes. Signed owner’s consent was obtained in all cases arriving to the Veterinary Teaching Hospitals of Extremadura and Murcia for including the animals in the study.
Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are not publicly due to legal reasons but available from the corresponding authors on reasonable request.

Competing interest

The authors declare that they have no competing interest.

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Authors’ contributions

MDCA, FT, DE and JJC conceived and designed the study. MDCA, AT, IM, MMC, DE and LF obtained the samples from the animals. MDCA, FT and DE analyzed the patient data. MDCA, FT, JJC and DE interpreted the patient data and drafted the manuscript. All authors critically read and edited the manuscript. All authors approved the final manuscript.

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Figure 1

Adenosine deaminase (ADA) activity from healthy animals. Serum (solid circle) and saliva (empty square) from dogs (A, n = 5), horses (B, n = 5), pigs (C, n=5) and cows (D, n=5), before and after adding erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) to the samples at different concentrations. Letters indicate
statistically significant results with the original samples (a: $P < 0.05$; b: $P < 0.01$; c: $P < 0.001$). Greek symbols indicate statistically significant results with the 0.1mM EHNA concentration ($\alpha$: $P < 0.05$). Horizontal dotted line shows the lower limit of quantification according to previously reported results [22, 28].

Figure 2
Linear regression between adenosine deaminase 2 (ADA2) results with manual vs. automated inhibition. Empty circles show pair of results obtained in serum (A) and saliva (B) when EHNA was added to samples at 4.0mM (S-EHNA) vs. when EHNA was added to the commercial reagent 1 at 0.80mM (R1-EHNA). Samples from 33 pigs (15 apparently healthy and 18 with disease) were used. The continuous line shows linear regression and the dotted lines show the 95% confidence interval. R2: Coefficient of linear regression.
Bland-Altman plot between adenosine deaminase 2 (ADA2) results with manual vs. automated inhibition. Empty circles show pair of results obtained in serum (A) and saliva (B) when EHNA was added to samples at 4.0mM (S-EHNA) vs. when EHNA was added to the commercial reagent 1 at 0.80mM (R1-EHNA). Samples from 33 pigs (15 apparently healthy and 18 with disease) were used. Separated lines show bias and dotted lines the 95% limits of agreement.