J. Vet. Med. A **50,** 72–78 (2003)
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ISSN 0931–184X

PHYSIOLOGY

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Determination of Autoantibodies to Thyroglobulin, Thyroxine and Triiodothyronine in Canine Serum

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With 1 figure and 3 tables

Received for publication: April 12, 2002

Summary

Enzyme immunoassays (EIAs) for the determination of autoanti-bodies (AA) to thyroid antigens in canine serum were developed. Streptavidin (SA) was immobilized as capture molecule on microtitreplates (MTP). Thyroglobulin (Tg) purified from canine thyroids and the thyroid hormones thyroxine and triiodothyronine (T3 and T4) were conjugated to biotin labelling reagents and attached to the MTP over the SA–biotin bridge. Bound AA were detected with anti-dog-immunoglobulin G (IgG) labelled with horseradish peroxidase. Serum samples from dogs which were allotted to four groups were analysed: A ($n = 31$), biochemical evidence of hypothyroidism; B ($n = 76$), clinical signs of hypothyroidism; C ($n = 47$), euthyroid with non-thyroidal disease; D ($n = 186$), clinically healthy. The validity of the assays was tested with two different methods. After thiophilic absorption chromatography of positive sera, a positive reaction in the EIA was only detected in those fractions which coeluted with the canine IgG standard. Furthermore, the positive reaction was blocked by the addition of the corresponding antigen. In 55% of the hypothyroid dogs AA to Tg and/or T3 and T4, respectively, were found (up to a titre of 1 : 1600). In group B 34% of the dogs were diagnosed positive, but the titre was lower (up to 1 : 400). In the groups C and D the number of dogs with AA and their titre was significantly lower. Two different methods for distinguishing positive and negative test results were compared in order to increase the specificity of the tests without decreasing the sensitivity. The EIAs are precise and based on high agreement with previous reported assays able to discriminate dogs with thyroiditis from healthy ones. These assays represent a good alternative to the isotope assays generally used for the analysis of AA to T4 and T3.

Introduction

Hypothyroidism is the most common endocrine disease in dogs. Primary hypothyroidism accounts for more than 95% of naturally occurring thyroid failure (Feldman and Nelson, 1996). One of the main causes of primary hypothyroidism is lymphocytic thyroiditis (LT), which represents an auto-immune-mediated destructive process. It is characterized by lymphocytic infiltration, multifocal and diffuse infiltrates of T-cells as well as plasma cells (Gosselin et al., 1981). Cytotoxic T-cells lead to destruction of thyrocytes, which are replaced by fibrous connective tissue. Circulating autoantibodies (AA) against thyroid antigens like thyroglobulin (Tg) and the thyroid hormones thyroxine (T4) and triiodothyronine (T3) are evident (Haines et al., 1984; Nachreiner et al., 1990; Beale, 1991). The opinions about the origin of AA to T3 and T4 (T3AA and T4AA) are controversial. Thyroid hormones are haptens and unable to induce antibody production. They have been considered as subsets of AA to Tg (TgAA) directed against hormonogenic sites (T3 or T4 containing epitopes; Young et al., 1988) or other proteins like thyroid hormone binding proteins or nuclear proteins which may act as the conjugating protein (Sakata, 1994). Their frequency in auto-immune-mediated thyroiditis is lower than that of TgAA, but they are more common in hypothyroid dogs. This suggests that dogs with more than one AA may have an increased risk of developing hypothyroidism compared with dogs with TgAA only (Graham et al., 2001). Additionally, AA can interfere with immunoassays used to measure the plasma concentration of T4 or T3.

Different methods have been established to detect circulating TgAA. The sensitivity of these assays varied within a wide range. The most constant and comparable results were obtained with enzyme immunoassays (EIAs). The predominant purpose of all methods is to reduce false positive results among healthy dogs and dogs with non-thyroidal illness without reducing the sensitivity of the test. The only commercially available EIA for canine TgAA (Oxford Biomedical Research, Inc. (OBR), MI, USA) was evaluated and a diagnostic threshold was determined (Nachreiner et al., 1998). The assay was found to be useful for the identification of LT after adapting the mode of calculation, which reduced the number of false positive results without changing the sensitivity.

Alongside T4, free T4 (estimated with equilibrium dialysis) and cTSH (Dixon and Mooney, 1999), the analysis of TgAA comprises additional helpful diagnostic information in canine thyroid disease. The detection of T3AA and T4AA contributes to the diagnosis and to the correct interpretation of the measured hormone concentrations that do not match clinical signs. However, for the methods described for the determination of thyroid hormone AA (THAA) the use of [^{125}I]-labelled T4 and T3 is necessary (Young et al., 1988; Rajatanavin et al., 1989). The major disadvantages of [^{125}I] are the short half-life and the restriction to specific laboratories, which hold the approval for isotope use.

The aims of this study were to establish EIAs with increased reproducibility and sensitivity for AA to thyroid antigens, to avoid radiolabelled chemicals, and to develop a simple method, which is practicable for clinical routine analysis for the estimation of T4AA and T3AA.

Materials and Methods

Enzyme immunoassays

Microtitreplates (MTP; 96 wells, maxisorp; Nunc, Nalgene-Nunc, Intern.) were coated with streptavidin (SA) (Sigma, Sigma-Aldrich, Austria; 0.5 μg/0.15 ml/well in sodium bicarbonate buffer, 0.05 mmol/l, pH 9.6) at room temperature (RT) overnight. Free binding sites were blocked with 0.2 ml of 1% bovine serum albumin (BSA) in TRIS buffered solution (TBS; 20 mmol/l, pH 7.5; NaCl 0.3 mol/l and 0.1% Tween 80) for at least 4 h at RT. The MTP were then emptied and stored sealed with parafilm at −20°C. The assays were performed in four steps. Prior to each step the MTP were washed four times with 0.02% Tween 20 using a microplate washer. All incubations were carried out on a microplate shaker at $100 \times \boldsymbol{g}$. First, 0.1 ml/well of biotinylated T3, T4 (0.5 μg/ml) and Tg (0.1 μg/ml) were bound on separate MTP for 1.5 h at RT. The biotinylated antigens were diluted in TBS containing 0.1% BSA. For the next step 0.1 ml of samples and controls diluted 1 : 100 with TBS containing 0.5% BSA and 8 μg/l of Anilino-naphthalene–sulphonic acid (Sigma) were incubated overnight at 4°C. In the third step 0.1 ml of anti-dog-immunoglobulin G(IgG) labelled with horseradish peroxidase (Sigma) diluted in TBS (0.1% BSA added) was incubated for 1 h at 4°C. Finally, 0.25 ml of the chromogen substrate (0.13 μmol/l tetramethyl-benzidine (Fluka, Sigma-Aldrich, Austria) and 0.13 mmol/l H_2O_2 in sodium acetate buffer 10 mmol/l, pH 5.0, with 5% citric acid) was added to each well. The colour reaction was stopped with 0.05 ml H_2SO_4 (2 mol/l) after 45 min and optical density (OD) was measured at 450 and 670 nm.

The test for canine Tg AA from OBR was performed according to the manufacturer's instructions.

Preparation and biotinylation of thyroglobulin

Thyroids were taken from healthy dogs of different breeds, which died of an accident. Thyroids were removed shortly after death and kept frozen at −20°C. A small piece of each gland was fixed in formalin for histological examination and only thyroids with normal histological appearance were used. The purification was carried out as described by Ericsson et al. (1984). The Tg containing fractions were re-chromatographed on Con A Sepharose 4B (Tarutani and Ui, 1985). Subsequently, the purity was evaluated on 6% SDS–PAGE under reducing and non-reducing conditions. Tg was conjugated with biotin–amido-caproate-sulpho-N-hydroxysuccinimide ester (Sigma) according to the protocol of the biotinylation kit (Sigma). The purified conjugate was aliquoted, lyophilized and stored at −20°C.

Biotinylation of T3 and T4

The carboxy group of the thyroid hormones was linked to the amino group of biocytin using a mixed anhydride reaction (Kellie et al., 1975). All steps were carried out as described by Palme and Möstl (1993) for the conjugation of steroid hormones to biotinylation reagents but the reaction temperature was changed to 4°C.

The hormone–biotin conjugates were separated by a preparative RP 18 chromatographic system (Lichroprep RP-18; Merck, Darmstadt, Germany; flow rate 4 ml/min) with a linear water–methanol gradient increasing from 30 to 90% methanol within 186 min; 76 fractions, each of 12 ml, were collected. An aliquot of each fraction was tested in an EIA. Briefly, MTP were coated with anti-rabbit IgG and free binding sites were blocked with 1% BSA. The biotinylated hormones were incubated together with an antibody against T3 or T4, respectively, both raised in rabbits (Sigma). After incubation and washing HRP-labelled SA was applied to the plates, which were incubated and washed again. The biotinylated products were identified by a substrate reaction with tetramethyl-benzidine. The relevant fractions were concentrated by passing through a primed C 18 cartridge, aliquoted and stored lyophilized at −20°C.

The working dilution of the biotinylated labels was estimated in a checkerboard titration comparing different dilutions of the biotinylated products with different dilutions of the second antibody in order to find the optimal dilutions of the biotinylated products. Parameters for comparison were the OD of the non-specific binding (NSB), which had to be as low as possible, and the OD difference between the negative and the positive control (PC) serum, which had to be as high as possible.

Controls and calculation of results

Serum samples were analysed in duplicates, PC and NSB in quadruplicates and the negative control (NC) in octruplicates. All controls were analysed in each run. The NC was a pool of sera from healthy dogs of various breeds, which were found to have normal T4 concentrations. NSB was defined as the background of binding by omitting the addition of dog sera and its OD was subtracted from all raw data before any further calculation. The PC was a pool of serum samples from dogs with autoimmune thyroiditis [donated by Professor Dr R. Nachreiner, Michigan State University (MSU), MI, USA]. Initially, all samples were diluted 1 : 100 for AA analysis. Positive sera were re-examined for estimation of the AA-titre. Sera were diluted in doubling dilutions till 1 : 1600 starting at 1 : 100. To improve the specificity of the tests without decreasing the sensitivity two methods for calculating the results were compared. For the first method (M I) the mean and the SD of the NC in each assay were determined. Samples were diagnosed as negative if their OD was less or equal to the NC + 3SD. For the second method (M II) the OD values from all healthy dogs were expressed as a percentage of the NC

in the respective run and the mean + 2SD of all these results was calculated. This value was slightly different in the three EIAs (158% for the TgAA, 150% for the T3AA and 143% for the T4AA). Therefore, an approximation was performed in order to make the calculation easier. A common threshold of 150% of the NC was taken to discriminate between positive and negative test results.

Accuracy

Analytical imprecision was tested by repeated analysis of samples with different AA titre. The intra-assay coefficient of variation (CV) was calculated from negative, low and high positive sera by analysing the same specimen at least 15 times in the same run. The inter-assay CV was calculated from the NSB and the NC and PC sera of 15–20 different runs. All calculations were based on OD [row data for NSB and transformed data (OD–OD of NSB) for the NC and PC].

Assay specificity

Specificity of the AA was tested by addition of T3, T4 and Tg (10, 100, 1000 and 10 000 ng/ml), respectively, to positive sera from hypothyroid dogs (the highest titre was used). The samples were incubated with the antigens overnight at 4°C with gentle shaking prior to analysis. Each displacement trial was tested in each assay.

To demonstrate that the binding agent was from the IgG fraction, 0.05 ml of positive sera diluted in 1 ml of sodium phosphate buffer (20 mmol/l, pH 6.8) containing ammonium sulphate (0.8 mol/l) was applied on thiophilic absorption chromatography [Fractogel EMD AF TA 650 (S); Merck; 50 × 10 mm]. The samples were eluted by a linear gradient of decreasing ammonium sulphate concentration (0.8 mol/l = 100%). Flow rate was 1 ml/min, 76 fractions of 1 ml were collected and the protein concentration of the eluate was measured continuously at 280 nm. Canine IgG (Chemicon, Intern. Inc.) was used as standard. Each fraction was analysed for binding capacity in the AA assays.

Serum samples

Serum samples which were received from clinics and practitioners were kept frozen at 20°C till analysis. Dogs of various breeds, ages, and both sexes were allotted to four groups. Group A: dogs with biochemical evidence of hypothyroidism (i.e. low serum T4-concentration and high cTSH value and/or negative response to TSH-stimulation test; $n = 31$). Group B: dogs with clinical signs of hypothyroidism such as lethargy, obesity, hyperlipidaemia, skin disorders, neuropathy, bradycardia or low voltage electrocardiogram but conflicting results in thyroid hormone and cTSH analysis ($n = 76$). Clinical chemistry and hormone analysis from group A and B dogs were mostly performed before submitting the samples for AA determination. These results and the case history were provided by the practitioners. Group C: dogs with other diseases (diagnosed and treated at the 1. medical clinic at the University of Veterinary Medicine of Vienna), which did not express clinical signs of endocrine dysfunctions, and dogs with skin diseases were excluded ($n = 47$). Group D: mainly Beagles, Huskies and Rottweiler ($n = 186$) which were considered as healthy after clinical examination and analysis of clinical chemical and haematological parameters (standard profiles). The samples of Huskies and Rottweiler were made available by another project in which clinical chemical and haematological parameters (standard profiles) of Huskies were compared with those of other breeds.

Statistical calculations

Analytical imprecision was calculated with routine descriptive statistical methods. Significant difference in prevalence and TgAA titre between the four groups of dogs was tested with ANOVA. For assessment of a group to group difference the Kruskall–Wallis ANOVA on ranks was used. The Wilcoxon signed rank test for paired observations was conducted to compare the results obtained with the two methods of calculating the cut-off value.

Results

Preparation of Tg and biotinylation of Tg, T3 and T4

No proteins besides Tg were detected by PAGE under reducing as well as non-reducing conditions.

Biotinylated T3 eluted in the fractions 48–49 and the T4-biotin conjugate eluted in the fractions 51–53. The optimal dilution for all labels was 1 : 2000, thus providing enough material for 800 MTP, which could be analysed with the yield of one coupling reaction. The biotinylated hormones could be stored frozen at −20°C for at least 2 years without loss of activity.

Validation of the assays

The results of the intra- and inter-assay CV are shown in Table 1. Addition of Tg to positive dog serum reduced the binding in the TgAA assay. Depending on the sample, the OD decreased below the values of the NC with Tg additions of 100–1000 ng/ml. Similar results were obtained for T3AA by addition of T3 in the T3AA EIA and for T4AA by addition of T4 in the T4AA EIA. The thyroid hormones decreased the titre of TgAA in some sera, but were not able to suppress the titre completely. Tg addition to sera positive for THAA only reduced the titre, if the Tg concentration was at least 10 000 ng/ml.

Table 1. Values of the intra- and inter-assay variation. For the intra-assay validation a negative serum, a low positive and a high positive serum were used. Parameters for the inter-assay validation were the non-specific binding (NSB) and a negative and a positive control serum. All calculations were based on optical density (OD) [row data for NSB and transformed data (OD–OD of NSB) for the controls]

	Intra-assay CV			Inter-assay CV		
Pool	Negative	High positive	Low positive	NSB	Negative	High positive
EIA for						
TgAA	3.2	3.4	5.3	14.2	17.4	10.6
T3AA	2.5	3.1	4.5	16.9	10.1	12.3
T4AA	5.6	7.0	10.0	16.6	18.7	17.1

CV, coefficient of variation; EIA, enzyme immunoassay; TgAA, autoantibodies to thyroglobulin; T3AA, autoantibodies to triiodothyronine; T4AA, autoantibodies to thyroxine.

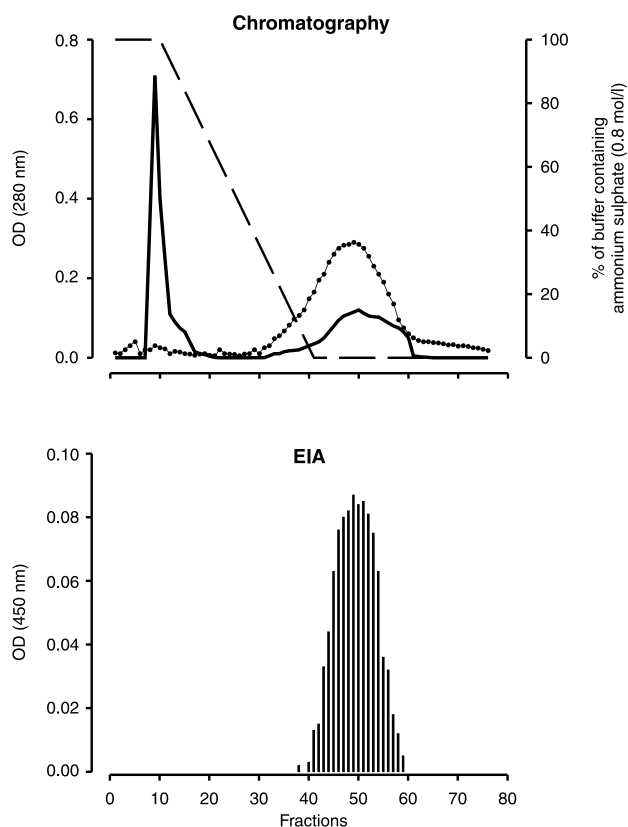

Fig. 1. Results of a thiophilic adsorption chromatography. The upper part of the figure shows the elution profile of the IgG-standard (• • •) and of a positive serum sample (——) detected at 280 nm. The dashed line (– – –) marks the decreasing ammonium sulphate concentration in the elution buffer. The lower part of the figure shows the binding of the fractions in the EIA for TgAA expressed as OD.

After the separation of the serum proteins of positive samples with thiophilic adsorption chromatography, a positive reaction in the TgAA, T4AA, and T3AA EIAs was only obtained in those fractions which coeluted with the canine IgG-standard (Fig. 1). The results of 30 samples in the present TgAA EIA were compared with the commercially available test from OBR: 80.6% were positive in both tests, 6.5% were positive in the present test only and 12.9% in the OBR test only. For the validation of the THAA–EIAs the results were compared with those of the isotope binding assay (Young et al., 1988) in 50 dog sera. The sera were provided by Professor Nachreiner and the isotope binding assay was performed at the MSU.

A concordance of 91.5% was found for the T3AA-test and of 97% for the T4AA-test, respectively. Mismatching results were localized within the low positive range of both assays.

Serum samples

Table 2 displays the prevalences of positive test results by applying the two methods for calculating the cut-off value to discriminate positive from negative sera. As the cut-off of M II is at a higher level the number of positive dogs decreased compared with the cut-off of M I. However, in group A only one dog changed to a negative test result. In group B the number of positives was 25% less and in the groups C and D a reduction in the number of positive dogs by an average of 57% occurred. Fewer dogs with non-thyroidal illness and fewer normal dogs found being positive indicated that by applying the cut-off of M II the specificity was enhanced without a reduction in the sensitivity of the EIAs. With M II a significant difference ($P = 0.004$) between group B and group C was noticeable, which was not evident by the use of M I. Therefore, the M II cut-off was chosen for further calculations of the test results.

Table 2. Comparison of the two methods for calculating the cut-off value. The mean + 3SD of the negative control was used for M I and in M II the cut-off was 150% of the negative control. The prevalence of positive test results for TgAA with or without thyroid hormone autoantibodies (THAA) and of a positive test result in any of the three enzyme immunoassays (EIAs) is expressed in percentage of the total number of dogs in each group

	Positive for TgAA alone or in combination with any other AA		Positive for at least one AA	
Cut-off	M I	M II	M I	M II
Group A	55.0	52.0	58.0	55.0
Group B	31.5	23.5	43.5	34.0
Group C	15.0	6.5	27.5	13.0
Group D	11.5	4.5	19.0	8.0

TgAA, autoantibodies to thyroglobulin; AA, autoantibodies; M I, first method; M II, second method.

The prevalence of positive reactions obtained with M II for the three analysed AA in each group is shown in Table 3. The majority of positive results was found with the EIA for TgAA. In 12 of the 16 positive samples in group A and in five dogs of group B (five of 18) the titre measured for TgAA was 1 : 400 to 1 : 1600. Whereas in the other groups titres of 1 : 200 to 1 : 400 were found in only one dog of group C (one of three) and D (one of nine), respectively. Focusing on the prevalence of TgAA an overall significant difference was ascertained ($P < 0.001$) between all groups. If the groups were compared pairwise a significant difference ($P < 0.05$ to 0.001) was evident with exception of the groups C and D. Comparing the titres of TgAA a similar result was obtained.

The THAA were determined in the groups A and B with a titre of 1 : 100 to 1 : 200; only three animals of group A had a titre of more than 1 : 400. The few positive results obtained in the groups C and D had a titre of just 1 : 100. A statistical analysis of these data was renounced because of the low prevalence of the THAA.

Discussion

The newly developed EIAs showed a good intra-assay precision with CV of less than 10%. The relatively high inter-assay CV of 10–20% partly originated in the low OD measured for some parameters. Additionally, factors such as the batch of buffer for the assay and the substrate reaction may alter the OD. In EIAs, in which the calculation was based on the relative binding, the intra-assay imprecision was lower, because the ratio of ODs was more constant than the day-to-day deviation of untransformed OD values. To evaluate the correspondence of the results gained with the newly developed assays with other findings, 30 sera were additionally analysed

Table 3. Prevalence of positive test results for TgAA, T3AA and T4AA in the four groups of dogs investigated. The results are expressed as percentage of the total number of dogs in each group which is written in brackets. For cut-off calculation the second method (M II) [150% negative control (NC)] was used

Groups of dogs Number of dogs	A 31		B 76		C 47		D 186	
Incidence of								
TgAA only	19.5	(6)	6.5	(5)	6.5	(3)	3.0	(6)
T3AA only	3.0	(1)	6.5	(5)	6.5	(3)	2.5	(5)
T4AA only	0.0		4.0	(3)	0.0		0.5	(1)
Combined incidence of								
TgAA, T3AA and T4AA	6.5	(2)	9.0	(7)	0.0		1.0	(2)
TgAA and T3AA	13.0	(4)	2.5	(2)	0.0		0.0	
TgAA and T4AA	13.0	(4)	5.5	(4)	0.0		0.5	(1)
T3AA and T4AA	0.0		0.0		0.0		0.0	
Positive for TgAA alone or in combination with any other AA	51.5	(16)	23.5	(18)	6.5	(3)	5.0	(9)
Positive for at least one AA	55.0	(17)	34.0	(26)	13.0	(6)	8.0	(15)

AA, autoantibodies; TgAA, autoantibodies to thyroglobulin; T3AA, autoantibodies to triiodothyronine; T4AA, autoantibodies to thyroxine.

in the assay from OBR and more than 80% conformable results for TgAA were found. A similar relationship was obtained for the T3AA and the T4AA. Mismatching results were only observed in the low positive range of the assays and the number of sera classified as positive in only one assay was distributed equally among the compared tests.

The specificity of the assays was verified by a significant reduction of the OD in the corresponding assays, if positive sera were incubated with increasing concentrations of antigen (Tg, T3 or T4) before analysis. In some sera, TgAA activity was also diminished by addition of T3 or T4. This agrees with another study (Gaschen et al., 1993) where the activity of TgAA was significantly reduced by the addition of free T3. However, the amount of inhibition did not correlate with the AA titre. In contrast, in other experiments, TgAA could not be inhibited by addition of thyroid hormones (Rajatanavin et al., 1989; Young et al., 1991; Thacker et al., 1992). For a better characterization of the T3AA, their binding to Tg was tested and a relatively high affinity constant (2.3×10^{-9}/M) was found. Therefore, it was concluded that the Tg molecule is involved in T3AA formation (Young et al., 1991). The different results in the various inhibition experiments may be due to the polyclonal nature of the thyroid AA, which can exhibit varying affinity and avidity in epitope reaction from animal to animal, as also described for human thyroid AA (Feldt–Rasmussen, 1996). Unfortunately, there was not enough serum from each patient to test the specific reactions of the thyroid AA in all our cases.

In addition to the validity for a distinct antigen, the validity of the binding agent was demonstrated. In thiophilic adsorption chromatography the IgG fraction of sera was separated from other serum proteins. Only in the fractions, which coeluted with the canine IgG-standard, a positive signal was measured in the EIA.

The performance of these assays with SA as the capture molecule provides many advantages over direct absorption of target proteins on the MTP. Direct binding of a protein can result in conformational changes and thus reduction of affinity to the analyte. Furthermore, active binding sites can become unavailable due to steric hindrance (Wild, 2001). In order to overcome this problem, SA was immobilized as a common capture molecule on the polystyrene surface. The affinity of AA to an antigen is much less reduced by its biotinylation than by its direct adsorption. Nearly all epitopes remain available for the AA by binding the protein with a SA–biotin bridge to the MTP (Dent, 2001). Additionally, haptens like the thyroid hormones cannot be fixed to the MTP by a direct immobilization procedure. This was the first time that T3AA and T4AA were analysed without radiolabelled tracers. The avoidance of radioactivity in laboratory practice was a success and enables laboratories not equipped for working with radioactive material to perform these specific examinations. The capture molecule provided a more constant distribution of the antigens on the MTP. In competition EIAs for steroid analysis it was demonstrated (Meyer, 1989) that the use of a capture anti-body decreased the intra-assay CV from $>10\%$ to $<5\%$. This enhanced the reproducibility and sensitivity of the EIAs. Moreover, the amount of antigens needed for an assay was much less compared with direct immobilization. For these TgAA–ELISAs concentrations of Tg ranging from 0.2 (Haines et al., 1984) to 4 μg/well (Young et al., 1991) were used. Whereas with the present assay a concentration of 0.01 μg/well was sufficient. Especially for proteins, which have to be purified from tissues of pets, it is important to use material saving methods as source materials may be difficult to obtain.

The additional analysis of THAA has the potential to increase the specificity of AA testing. The prevalence of a combined occurrence of TgAA and at least one THAA was much higher in the groups A and B. In the group of healthy dogs two animals were found positive for all three AA analysed and in one dog TgAA were combined with T4AA, but there was no possibility to re-examine these cases some months later. In another study healthy dogs with a combined incidence of TgAA and THAA were found to have a slightly increased risk of developing thyroid dysfunction compared with those which had isolated TgAA (Graham et al., 2001). However, further studies about the predictive value for a clinically healthy dog with AA to Tg and TH for becoming hypothyroid are warranted.

For estimating THAA various techniques (Rajatanavin et al., 1989; Young et al., 1991; Thacker et al., 1992; Gaschen et al., 1993) have been used and the results concerning the prevalence of THAA deviated within a wide range. However, all studies gave evidence about their importance for the interpretation of T4 and T3 serum concentrations not matching the clinical signs. THAA can interfere with the anti-bodies in competition assays used for the determination of T4 and T3

concentrations in canine sera, leading to artificial high or low values, depending on assay technology.

In the group of dogs with biochemical evidence of hypothyroidism the number of positive results for TgAA was comparable with other studies (Haines et al., 1984; Beale et al., 1990; Thacker et al., 1992; Dixon and Mooney, 1999) which found prevalences ranging from 36 to 59%. All but four of these dogs (12 of 16) had a titre between 1 : 400 and 1 : 1600 for TgAA and more than half of them (10 of 16) had T3AA and/or T4AA as well. T3AA were found to be common in hypothyroid dogs with TgAA (Nachreiner et al., 1998; Graham et al., 2001) and their frequency is higher than that of T4AA.

It is remarkable that in the groups B and C 6.5% of the dogs presented T3AA without having TgAA. Whereas in the groups A and D only 3% of the dogs showed isolated T3AA. Only T4AA were found in dogs of group B and D, but their prevalence was quite low (4 and 0.5%, respectively). The occurrence of isolated THAA was described for a minority of dogs but they were not associated with signs of hypothyroidism (Thacker et al., 1992). This was not found in every case in the current study, as isolated T3AA or T4AA were also estimated in dogs of group B, which were suspected of thyroid disorder and in dogs of group A, which showed biochemical evidence of hypothyroidism. However, in humans, their frequency is higher and they were found in patients with and without thyroid disorders, but their origin and significance are not fully understood (Sakata, 1994).

In one study (Nachreiner et al., 1998), the prevalence of false positive results was markedly reduced without decreasing the sensitivity of the test by an alternative calculation of the cut-off value. Instead of using two or three SDs above the mean OD of the NC serum as the threshold for a positive result, as described in some reports (Haines et al., 1984 Young et al., 1991; Thacker et al., 1992), a cut-off of two times the mean OD of the NC serum was used to distinguish positive test results. The definition of a negative result was still within two SDs of the NC but an inconclusive range, between definite positive and definite negative results was introduced. A similar observation was made with this study. Using the common and repeatedly described method for calculating a cut-off value (M I) a notable number of positive test results was found in the groups of dogs without hypothyroidism and suspected of hypothyroidism. As the intra-assay CV of the NC was very low, this type of cut-off value produced many false positive test results, thus reducing the specificity of the test. With this cut-off a significant distinction between dogs with any non-endocrine disease and dogs suspected of hypothyroidism was not possible. To overcome this problem the cut-off was calculated by using a large number of healthy dogs. The ODs of the samples were expressed as ratios of the NC. The 95% confidence intervals with this mode of evaluation were between 143 and 158% of the NC in the three EIAs. In order to simplify the calculation a cut-off of 150% of the NC was chosen for all EIAs. Applying this method of calculation, the number of positive test results was significantly reduced in the groups B, C and D. However, the prevalence of positive results in group A remained nearly unchanged. Additionally, a significant difference between the groups B and C was then evident.

It is not surprising that there was no significant difference in the prevalence of AA between the groups A and B. Dogs suspected of being hypothyroid for clinical signs may often still have low normal thyroid hormone concentrations, but as LT is the main course of thyroid destruction, AA can be found at these early stages of the disease. AA are well associated with lymphocytic infiltrations. In 10 of 11 hypothyroid dogs positive for TgAA, mononuclear cells infiltrating the thyroid gland were found in biopsies (Iversen et al., 1998). Also not all positive results among the healthy dogs are false positive results. In a study of 171 healthy but AA positive dogs examined over a 1-year period, 4% became hypothyroid and 13% exhibited subclinical hypothyroidism (Graham et al., 2001). An earlier study also gave evidence of low or limited disease progression of AA positive dogs (Haines et al., 1984). Even pathological changes did not imply functional abnormalities (Graham et al., 2001). Therefore, with the first detection of AA without decreased thyroid hormone concentrations, it is uncertain whether a dog will become hypothyroid or not.

In conclusion, these SA–biotin-based EIAs were a reliable tool for the estimation of AA to thyroid antigens in canine sera. In comparison with direct immunoassays, the use of SA as capture molecule provided four main advantages. First, the necessary amount of Tg was diminished to 1% of that needed for direct coating. Secondly, the biotinylated labels used for T3AA and T4AA analysis were stable for more than 2 years without reduction of activity. Thirdly, all AA to thyroid antigens could be analysed with the same technique. And fourthly, the prevalence of AA matched well with previous reports.

Acknowledgements

The authors thank Professor Dr R. Nachreiner, Michigan State University, USA for the gift of dog sera and results of THAA analysis and valuable discussions of the manuscript, the 1. medical clinic for small animal and horses of the University of Veterinary Medicine, Vienna, for submission of dog sera, Mag. Andreas Jerzö for providing the serum samples of healthy Rottweilers and Huskies and DI. Wanda Kawinek for technical assistance. Parts of this study were supported by the Hochschuljubiläumsstiftung der Stadt Wien (H-00213/94).

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