Detection of autoantibodies against thyroid peroxidase in serum samples of hypothyroid dogs

Evelyn Skopek, DVM; Martina Patzl, DVM; Raymond F. Nachreiner, DVM, PhD

Objective—To establish a sensitive test for the detection of autoantibodies against thyroid peroxidase (TPO) in canine serum samples.

Sample Population—365 serum samples from dogs with hypothyroidism as determined on the basis of serum concentrations of total and free triiodothyronine (T_3), total and free thyroxine (T_4), and thyroid-stimulating hormone, of which 195 (53%) had positive results for at least 1 of 3 thyroid autoantibodies (against thyroglobulin [Tg], T_4, or T_3) and serum samples from 28 healthy dogs (control samples).

Procedure—TPO was purified from canine thyroid glands by extraction with detergents, ultracentrifugation, and precipitation with ammonium sulfate. Screening for anti-TPO autoantibodies in canine sera was performed by use of an immunoblot assay. Thyroid extract containing TPO was separated electrophoretically, blotted, and probed with canine sera. Alkaline phosphatase–conjugated rabbit anti-dog IgG was used for detection of bound antibodies.

Results—TPO bands were observed at 110, 100, and 40 kd. Anti-TPO autoantibodies against the 40-kd fragment were detected in 33 (17%) sera of dogs with positive results for anti-Tg, anti-T_4, or anti-T_3 autoantibodies but not in sera of hypothyroid dogs without these autoantibodies or in sera of healthy dogs.

Conclusions and Clinical Relevance—The immunoblot assay was a sensitive and specific method for the detection of autoantibodies because it also provided information about the antigen. Anti-TPO autoantibodies were clearly detected in a fraction of hypothyroid dogs. The value of anti-TPO autoantibodies for use in early diagnosis of animals with thyroid gland diseases should be evaluated in additional studies. (*Am J Vet Res* 2006;67:809–814)

Abbreviations

TPO	Thyroid peroxidase
Tg	Thyroglobulin
T_3	Triiodothyronine
T_4	Thyroxine
TSH	Thyroid-stimulating hormone
aAb	Autoantibody
TBS	Tris-buffered saline

Formation of thyroid hormones in the thyroid gland is catalyzed by TPO. Membrane-bound TPO is a glycosylated hemoprotein enzyme that faces the closed colloid space and acts at the apical membrane of thyrocytes. It plays a central role in the biosynthesis of thyroid hormones by iodination of tyrosine residues of Tg and condensation of 2 iodotyrosyl residues into T_3 and T_4.

Hypothyroidism caused by lymphocytic thyroiditis is a common endocrine disease in humans and dogs. Lymphocytic thyroiditis and idiopathic follicular atrophy comprise the main causes of primary hypothyroidism in dogs and account for > 95% of the dogs with this disease.[1] Clinical signs of hypothyroidism are not evident until at least 75% of the thyroid gland tissue is destroyed. The diagnosis of hypothyroidism in dogs is made on the basis of appropriate clinical signs as well as low circulating concentrations of total or free T_4 and T_3 and an increase in the concentration of TSH. However, TSH concentrations are within the reference range in many hypothyroid dogs. In those cases, thyroid responsiveness to the administration of exogenous TSH should be evaluated.[2]

The pathogenesis of lymphocytic thyroiditis is commonly accepted to be an autoimmune process.[3] Autoimmune diseases are characterized as damage of tissues by autoreactive lymphocytes and antibodies directed against host tissues. The entire sequence of events leading to damage of the thyroid gland is not exactly understood. However, aAbs against TPO (ie, anti-TPO aAbs) are reportedly capable of fixing complement and are responsible for the T-cell–mediated cytotoxic effects against thyroid cells.[4] Apparently, anti-Tg aAbs do not have the ability to mediate cytotoxic effects.[5,6] In humans with autoimmune diseases of the thyroid gland, the major autoantigens are Tg and TPO. Therefore, tests for anti-Tg and anti-TPO aAbs are performed to diagnose thyroid gland diseases.[7] Lymphocytic thyroiditis in dogs is histologically and clinically comparable to Hashimoto's thyroiditis in humans.[3,8] Tests for anti-Tg, anti-T_4, and anti-T_3 aAbs are available,[9,10,a] and tests for anti-TPO aAbs have been described.

Various immunologic methods have been used to estimate concentrations of anti-TPO aAbs.[11-13] However, variable results have been reported[11-13] for dogs. In 1 study,[11] investigators described anti-TPO aAbs detectable by use of an ELISA in sera of 10 of 34 (29%)

Received July 29, 2005.
Accepted November 4, 2005.
From the Department of Natural Science, Institute of Biochemistry, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria (Skopek, Patzl); and the Diagnostic Center for Population and Animal Health, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48823 (Nachreiner). Dr. Patzl's present address is Clinical Department for Diagnostic Imaging, Infectious Diseases and Clinical Pathology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria.
Supported by the Hochschuljubiläumsstiftung der Stadt Wien (H212/2000).
The authors thank Professors Elmar Bamberg and Armin Saalmüller for technical assistance.
Address correspondence to Dr. Patzl.

clinically hypothyroid dogs. In contrast, investigators in another study[12] did not confirm those results and described a lack of anti-TPO aAbs in dogs as determined by analysis of sera from 50 hypothyroid dogs by use of anti-Tg, anti-T_4, or anti-T_3 aAbs. In a third study,[13] investigators identified anti-TPO aAbs by use of indirect immunofluorescence in 16 of 64 (25%) Beagles in a breeding colony. The dogs of that study did not have any clinical signs of hypothyroidism, but clinical variables were unfortunately not analyzed.

The objective of the study reported here was to overcome potential sensitivity problems in the test systems used to detect thyroid aAbs. Our intent was to establish a reliable test for the detection of anti-TPO aAbs in canine serum by use of purified TPO and the high sensitivity of immunoblot assays.

Materials and Methods

Sample population—Serum samples from 365 dogs were screened for anti-TPO aAbs. Serum samples had been submitted to the endocrinology laboratory at the Michigan State University College of Veterinary Medicine for diagnosis of thyroid gland disorders. Mean ± SD age (known for 318 dogs) was 5.7 ± 3.0 years. Sex was known for 330 dogs (58% females and 42% males). Breed was known for 330 dogs and included 59 (17.9%) Golden Retrievers, 43 (13.0%) mixed-breed dogs, 21 (6.4%) Labrador Retrievers, and 21 (6.4%) Cocker Spaniels; all other breeds were represented by < 16 (5.0%) dogs.

Concentrations of total T_3 and T_4, free T_3 and T_4, and TSH were within ranges consistent with hypothyroidism. Samples were grouped on the basis of concentrations of TSH and free T_4, which were considered to be the most important variables (**Appendix**). Concentrations of thyroid-specific aAbs (anti-Tg, anti-T_4, or anti-T_3 aAbs) were determined. Of the 365 samples, 195 (53%) yielded positive results for at least 1 of the thyroid-specific aAbs (anti-Tg aAbs only, 151; anti-Tg and anti-T_4 aAbs, 12; anti-Tg and anti-T_3 aAbs, 9; anti-Tg, anti-T_3, and anti-T_4 aAbs, 17; anti-T_4 aAb only, 3; and anti-T_3 and anti-T_4 aAbs, 3).

Control samples of sera from 28 healthy dogs (23 Huskies and 5 Rottweilers; mean ± SD age, 3.9 ± 2.6 years; 16 females and 12 males) were tested for anti-TPO aAbs. In a study[9] in which investigators used another screening test, these samples had negative results for anti-Tg, anti-T_4, and anti-T_3 aAbs.

Purification of TPO—Procedures for the purification of TPO have been reported for samples obtained from pigs,[14–17] cattle,[18] humans,[19–21] and dogs.[12] The purification procedure used for the study reported here has been described for human[19] and canine[12] TPO. Thyroid glands were obtained from 40 dogs (mean ± SD, 10.6 ± 2.4 years old) immediately after they were euthanized because of various diseases (heart insufficiency, renal failure, arthropathy, or infirmity). Thyroid glands were stored at –20°C. All steps of the isolation procedure were performed in a room that was cold (4°C) or an ice bath, unless stated otherwise. Buffers used were described in another study.[12]

Frozen thyroid glands were allowed to thaw slightly and then were sliced into small pieces. Blood was removed by 3 washes with cold NaCl solution (0.15 mol/L). For homogenization, Tris-HCl containing a protease inhibitor (benzamidine; 10 mmol/L) was added. Homogenate was filtered through gauze and centrifuged at 48,000 × *g* for 1 hour. The resulting pellet was suspended in buffer (Tris-HCl, 0.05 mol/L; KCl, 0.15 mol/L; and KI, 0.1 mmol/L [pH, 8.0]). For solubilization, homogenate was warmed to 24°C, and sodium deoxycholate (final concentration, 1%), Triton X-100 (final concentration, 1%), and crystalline trypsin (final concentration, 140 mg/L) were added in succession. The solution was vigorously stirred for 2.5 hours, and the process was then stopped by the addition of soybean trypsin inhibitor.[19]

The solution was again centrifuged (100,000 × *g* for 60 minutes), and the turbid, reddish-yellow supernatant was collected. The protein fraction was precipitated by addition of ammonium sulfate. The solution was vigorously stirred, which was followed by centrifugation (17,000 × *g* for 40 minutes at 4°C). Then, the floating precipitate was resuspended in buffer (Tris-HCl, 0.025 mol/L; KCl, 0.15 mol/L; and KI, 0.1 mmol/L [pH, 7.0]) and stirred for 30 minutes at 24°C. This suspension was centrifuged again at 100,000 × *g* for 60 minutes, and the resulting supernatant was dialyzed against buffer (Tris-HCl, 0.025 mol/L; and KI, 0.5 mmol/L [pH, 7.0]) for 16 hours at 4°C. The dialyzed solution was concentrated in an ultrafiltration cell[b] with a cutoff value of 10 kd.[12] The pellet was resuspended in buffer and dialyzed in a manner similar to that used for the supernatant. At each step of the purification procedure, an aliquot was obtained and used to determine the protein concentration[22] and TPO activity. Activity of TPO was determined on the basis of the ability to oxidize guaiacol.[12]

Identification of TPO and Tg—Several antibodies against human TPO and Tg were used to identify canine TPO and Tg. This panel of antibodies contained 2 monoclonal mouse anti-human TPO antibodies,[c,d] a polyclonal rabbit anti-human TPO antibody,[e] a monoclonal rabbit anti-human Tg aAb,[f] and a polyclonal rabbit anti-human Tg aAb.[g] Alkaline phosphatase–conjugated rabbit anti-dog IgG,[h] alkaline phosphatase–conjugated goat anti-mouse IgG,[i] and alkaline phosphatase–conjugated anti-rabbit IgG[j] were applied as second antibodies. Cross-reactivity was also tested by use of human TPO[k] as the antigen. A commercially available standard[l] was used for estimation of molecular weights.

Gel electrophoresis and immunoblot assay—The SDS-PAGE was performed for reducing and nonreducing conditions on an 8.5% cross-linked gel. After electrophoresis, the gel was stained with silver nitrate or was blotted onto a polyvinylidene difluoride membrane[m] by use of semidry blotting (discontinuous buffer system without methanol and SDS).[23] Free binding sites were blocked with TBS solution

Table 1—Amount of total protein and activity of canine TPO at various stages of the purification procedure.

		TPO	
Stage of purification	**Total protein (mg)**	**Activity (U/L)**	**Specific activity (U/g)**
Precipitate-centrifugation*	44.60	29.30	0.6
Precipitate–ammonium sulfate†	20.88	131.85	79.4
Supernatant after dialysis	11.58	51.26	88.6
Precipitate after dialysis	9.40	170.00	144.7

*Represents precipitate harvested after centrifugation at 48,000 × *g*. †Represents precipitate harvested after the addition of ammonium sulfate.

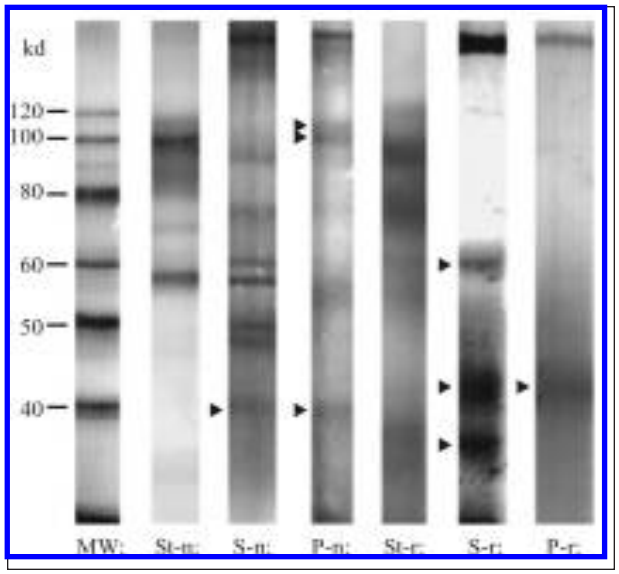

Figure 1—Photograph of an 8.5% cross-linked gel of canine thyroid gland samples after SDS-PAGE conducted for reducing (r) and nonreducing (n) conditions. Silver stain was used to confirm purity of TPO. MW = Molecular weight standards. St = Human TPO standard. S = Supernatant preparation. P = Precipitate preparation.

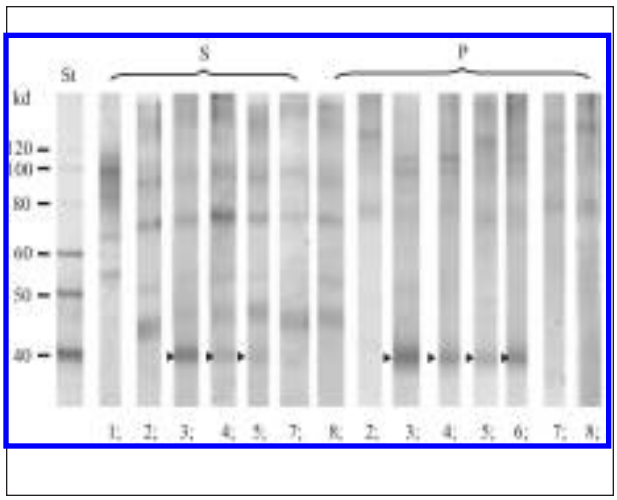

Figure 2—Immunoblot of purified human TPO incubated with monoclonal mouse anti-human TPO antibody (lane 1); supernatant preparations incubated without canine serum (nonspecific binding; lane 2) or with various canine sera that had positive (lanes 3–7) or negative (lane 8) results for thyroid-specific aAbs (anti-Tg, anti-T_4, or anti-T_3 aAbs; dilution, 1:100); and precipitate preparations incubated without canine serum (lane 2) or with various canine sera that had positive (lanes 3–7) or negative (lane 8) results for thyroid-specific aAbs. Notice the bands at 40 kd (arrowheads). *See* Figure 1 for remainder of key.

containing 5% skim milk powder, and the membrane was then cut into longitudinal strips and probed with serum samples diluted 1:100 in TBS solution containing Tween 20 and 5% skim milk powder. Bound aAbs were developed by use of alkaline phosphatase–conjugated rabbit anti-dog IgG,[h] with 5-brom-4-chlor-3-indolyl-phosphate (0.15 mg/mL) and nitroblue-tetrazolium (0.3 mg/mL) used for color reaction.[24] The binding of the antibodies, which were used as control samples or to determine their reactivity with TPO or Tg, was developed by use of alkaline phosphatase–conjugated goat anti-mouse IgG[i] or alkaline phosphatase–conjugated anti-rabbit IgG.[j] Nonspecific binding was evaluated by replacing the first antibody with buffer.

Results

Purification of TPO—High TPO activity was detected in the supernatant and precipitate after the second centrifugation step (100,000 × *g*) and dialysis (**Table 1**). Purity of the supernatant and precipitate preparations for nonreducing and reducing conditions was validated by use of a silver stain (**Figure 1**).

For nonreducing conditions, a TPO band was seen at 40 kd for the supernatant and precipitate preparations, and 2 additional bands were seen at 100 and 110 kd, respectively, for the precipitate preparation. All other bands, except albumin at approximately 57 kd, were identified by use of nonspecific binding as fragments of immunoglobulins. For reducing conditions, TPO bands were identified at approximately 60, 43, and 37 kd for the supernatant preparation and 43 kd for the precipitate preparation.

Anti-TPO aAbs—Sera from 33 of 195 dogs with anti-Tg, anti-T_4, or anti-T_3 aAbs bound to the 40-kd band of the supernatant preparation (**Figure 2**). However, many sera containing high titers for anti-Tg aAbs did not bind to the 40-kd band. Mean ± SD age of these 33 dogs was 3.9 ± 1.9 years, and 21 (64%) were females, and 12 (36%) were males. Golden Retrievers (17/33 [52%]) were the most prominent breed, despite the fact that they accounted for only 17.9% of the 365 dogs in the sample population. Prevalence for all other breeds was ≤ 6%.

Results for the 33 dogs with positive results for thyroid-specific aAbs were grouped on the basis of TSH and free T_4 concentrations. Twenty dogs had high concentrations of TSH and free T_4, 2 dogs had high concentrations of TSH but free T_4 concentrations within the reference range, 3 dogs had TSH concentrations within the reference range but free T_4 concentrations less than the reference range, and 8 dogs had concentrations of TSH and free T_4 within the respective reference ranges.

Several samples did not bind to the 40-kd band. The 170 serum samples that had negative results for thyroid-specific aAbs, the serum samples from the 28 healthy dogs, and the rabbit anti-human Tg antibody did not bind to the 40-kd band.

The 33 samples that had positive results with the 40-kd band for the supernatant preparation also reacted with the 40-kd band for the precipitate preparation. Furthermore, 10 of the 33 samples recognized proteins for the same preparations at 100 and 110 kd (Figure 2).

Nonspecific binding revealed bands at approximately 250, 180, 150, 90, 70, 52, and 47 kd for the supernatant preparation and 200, 140, and 80 kd for the precipitate preparation. These were identified as fragments of IgG on the basis that the second antibody (alkaline phosphatase–conjugated rabbit anti-dog IgG) bound directly when the specific antibody or canine serum, respectively, was replaced by buffer.

Western blot analysis revealed that the monoclonal and polyclonal antibodies to human TPO reacted with the human TPO standard, but there was no cross-reaction between these TPO antibodies and the preparations of canine TPO. As expected, the anti-human Tg antibodies did not bind to specific bands for human

and canine TPO (data not shown). The TPO preparations were also separated by use of reducing conditions and probed with the same serum samples, but there was not a discernable reaction with the reduced proteins (data not shown).

Discussion

Conformity is evident for autoimmune-mediated hypothyroidism in dogs and humans. The function of anti-TPO aAbs in destruction of the thyroid gland is not completely understood in humans and other animals. Several authors[4–6] described finding anti-TPO aAbs associated with the ability to fix complement and mediate cytotoxic effects against thyroid cells in vitro. This fact and the fact that anti-TPO aAbs were not consistently described or found by other groups of investigators[11,13,25] were the impetus for the study reported here.

Homogenization, several centrifugations, addition of detergents, and protein precipitation were used to purify TPO. Activity of TPO was estimated by oxidation of guaiacol,[12] and the results for the study reported here were similar to those reported in other studies.[15,19] However, a high amount of activity was also measurable in the precipitate preparation obtained after the second centrifugation step (100,000 × *g*). Analysis of that fraction was not described in either of 2 other studies.[12,19] Enzyme activity was measurable in that precipitate, and there were also specific protein bands (110, 100, and 40 kd for nonreducing conditions) detected by use of immunoblotting techniques. Identification of TPO in the precipitate preparation was an important finding that enhanced the yield of TPO.

In the study reported here, bands for canine TPO were evaluated by use of SDS-PAGE and a silver stain. For nonreducing conditions, bands were detected at 110, 100, and 40 kd, whereas bands were detected at 60, 43, and 37 kd for reducing conditions. The results obtained from nonreduced and reduced TPO fragments are the same as those for preparations of canine thyroid glands described in another study.[12] Furthermore, size of the TPO fragments is comparable to findings in humans[19,26] and pigs.[14,27-29]

In 33 of 195 (17%) canine sera that had positive results for at least 1 thyroid-specific aAb (anti-Tg, anti-T_4, or anti-T_3 aAbs), there was a reaction with the 40-kd band. A protein of that size has been identified as a fragment of TPO in thyroid gland preparations of dogs and pigs.[12,14] Sera from hypothyroid dogs without aAbs and sera from healthy dogs did not bind to that specific protein band. These results do not agree with findings reported elsewhere.[12] Furthermore, anti-TPO aAbs with titers between 1:100 and 1:1,425 were found in sera from 5 dogs with multiple autoimmune endocrinopathies.[25] In another study,[11] 34 sera from hypothyroid dogs were analyzed by use of an ELISA to detect aAbs against thyroid microsomal antigen (which corresponds to TPO), and titers of 1:20 to 1:1,280 were found in 10 (29%) of those samples. Other investigators[13] described use of indirect immunofluorescence to detect aAbs against canine TPO within the follicular epithelial cytoplasm and apical cell membranes. Those investigators found antimicrosomal antigen aAbs in 64 of 260 (25%) sera obtained from Beagles in a semi-closed breeding colony. Use of immunofluorescence may also result in detection of Tg because of the close proximity of the 2 proteins within the thyrocytes. This would explain the higher incidence of dogs with antimicrosomal antigen aAbs in that study.[13] False-positive results are also possible for an ELISA when the purity of the antigen preparation is not adequate. Therefore, immunoblotting techniques are a more sensitive and specific serologic method, and in addition, it provides information about the size of the antigenic proteins. Furthermore, use of electrophoresis to separate the protein extract enhances purity of the distinct fractions. This may explain why the percentage of positive results of anti-TPO aAbs by use of the immunoblotting assay is lower than for an ELISA or use of immunofluorescence.

Although lymphocytic thyroiditis in dogs is histologically and clinically comparable to Hashimoto's thyroiditis in humans, the number of positive results for anti-TPO aAbs in the sera of hypothyroid dogs is not comparable to that for human patients with Hashimoto's thyroiditis. In humans, the highest prevalence of anti-TPO aAbs was in patients with Hashimoto's thyroiditis who were also evaluated because of other thyroid gland dysfunction. The prevalence of anti-TPO aAbs in human patients with Hashimoto's thyroiditis depends on the test system.[30] In patients with Hashimoto's thyroiditis, aAbs against reduced TPO were found in 60% of patients, whereas aAbs against nonreduced TPO were detected in 76% of patients.[31] In another study,[30] up to 90% of patients with Hashimoto's thyroiditis had anti-TPO aAbs detected by use of an ELISA for anti-TPO. In the study reported here, the prevalence of anti-TPO aAbs in hypothyroid dogs was 17% by use of an immunoblotting technique. Other investigators detected anti-TPO aAbs in 29% of dogs by use of an ELISA[11] and in 25% of dogs by use of indirect immunofluorescence.[13] Analysis of these results, in combination with findings of another study[32] in humans, suggests that in contrast to humans, autoimmune thyroiditis in dogs does not appear to be induced or trigged only by anti-TPO aAbs. Investigators in 1 study[13] revealed a low correlation between serum TPO aAbs and histologic verification of lymphocytic thyroiditis. They discussed the possibility that the beginning of inflammatory changes and detection of serum aAbs was not a synchronous process. Therefore, anti-TPO aAbs in canine sera may appear earlier than lymphocytic thyroiditis.

However, there is also a study[28] in which investigators found that TPO is able to induce thyroiditis in domestic animals. That study revealed that thyroiditis could be successfully induced in rats by use of porcine TPO as an antigen. To prove that anti-TPO aAbs in dogs have the potential to induce lymphocytic thyroiditis, a study that combines tests for anti-TPO aAbs with histologic analysis of thyroid glands should be conducted.

Two other groups of dogs may be of interest for more detailed investigations. One group is dogs that have primary hypothyroidism but that lack anti-Tg aAbs (so-called idiopathic hypothyroidism). The second group is dogs < 5 years old that have thyroid-specific aAbs because approximately 20% will develop

thyroid gland dysfunction within 1 year after the detection of thyroid-specific aAbs.[n]

The immunoblot assay established for the detection of anti-TPO aAbs proved to be a sensitive test. Analysis of the results of the study reported here revealed detection of anti-TPO aAbs in dogs on the basis that immunoblot-specific bands of known molecular weight were detectable and false-positive results can be excluded. Dogs of various breeds, dogs at various stages of disease, and healthy dogs should be evaluated in a follow-up study to determine the predictive and diagnostic value of the test for anti-TPO aAbs in the diagnosis of hypothyroidism in dogs.

a. Nachreiner RF, Refsal KR, Thacker EL, et al. Incidence of T3 and T4 autoantibodies in dogs using a sensitive binding assay (abstr). *J Vet Intern Med* 1990;4:114.
b. Vivaspin 2, VIVASCIENCE, Hannover, Germany.
c. Monoclonal mouse anti-human thyroid peroxidase, DAKO, Glostrup, Denmark.
d. Monoclonal mouse anti-human thyroid peroxidase, HyTest, Turku, Finland.
e. Polyclonal rabbit anti-human thyroid peroxidase, BIOTREND, Köln, Germany.
f. Thyroglobulin Ab-6, NeoMarkers, Fremont, Calif.
g. Polyclonal rabbit anti-human thyroglobulin, DAKO, Glostrup, Denmark.
h. Anti-dog IgG (whole molecule) alkaline phosphatase conjugate, Sigma Chemical Co, St Louis, Mo.
i. Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate, Sigma Chemical Co, St Louis, Mo.
j. Alkaline phosphatase conjugated affinity purified anti-rabbit IgG (H&L) [goat], Rockland, Gilbertsville, Pa.
k. Thyroid peroxidase, US Biological, Swampscott, Mass.
l. MagicMark western standard, Invitrogen, Paisley, UK.
m. Immune-blot PVDF membrane, Bio-Rad, Hercules, Calif.
n. Graham PA, Lundquist RB, Refsal KR, et al. A 12-month prospective study of 234 thyroglobulin antibody positive dogs which had no laboratory evidence of thyroid dysfunction (abstr). *J Vet Intern Med* 2001;15:298.

References

1. Feldman EC, Nelson RW. Hypothyroidism. In: Feldman EC, Nelson RW, eds. *Canine and feline endocrinology and reproduction*. 2nd ed. Philadelphia: WB Saunders Co, 1996;187–265.
2. Panciera DL. Is it possible to diagnose canine hypothyroidism? *J Small Anim Pract* 1999;40:152–157.
3. Beale KM. Canine immune-mediated thyroiditis. *Vet Med Rep* 1991;3:123–130.
4. Stassi G, De Maria R. Autoimmune thyroid disease: new models of cell death in autoimmunity. *Nat Rev Immunol* 2002;2:195–204.
5. Guo J, Jaume JC, Rapoport B, et al. Recombinant thyroid peroxidase-specific Fab converted to immunoglobulin G (IgG) molecules: evidence for thyroid cell damage by IgG1, but not IgG4, autoantibodies. *J Clin Endocrinol Metab* 1997;82:925–931.
6. Bogner U, Schleusener H, Wall JR. Antibody-dependent cell mediated cytotoxicity against human thyroid cells in Hashimoto's thyroiditis but not Graves' disease. *J Clin Endocrinol Metab* 1984;59:734–738.
7. Duthoit C, Estienne V, Durand-Gorde JM, et al. Thyroglobulin monoclonal antibody cross-reacting with thyroperoxidase induces in syngeneic mice anti-idiotypic monoclonal antibodies with dual autoantigen binding properties. The intertope hypothesis. *Eur J Immunol* 1999;29:1626–1634.
8. Vajner L, Vortel V, Brejcha A. Lymphocytic thyroiditis in beagle dogs in a breeding colony: histological findings. *Vet Med (Praha)* 1997;42:43–49.
9. Patzl M, Möstl E. Determination of autoantibodies to thyroglobulin, thyroxine and triiodothyronine in canine serum. *J Vet Med A Physiol Pathol Clin Med* 2003;50:72–78.
10. Haines DM, Lording PM, Penhale WJ. Survey of thyroglobulin autoantibodies in dogs. *Am J Vet Res* 1984;45:1493–1497.
11. Haines DM, Lording PM, Penhale WJ. The detection of canine autoantibodies to thyroid antigens by enzyme-linked immunosorbent assay, hemagglutination and indirect immunofluorescence. *Can J Comp Med* 1984;48:262–267.
12. Thacker EL, Davis JM, Refsal KR, et al. Isolation of thyroid peroxidase and lack of autoantibodies to the enzyme in dogs with autoimmune thyroid disease. *Am J Vet Res* 1995;56:34–38.
13. Vajner L. Lymphocytic thyroiditis in beagle dogs in a breeding colony: findings of serum autoantibodies. *Vet Med (Praha)* 1997;42:333–338.
14. Taurog A, Lothrop ML, Estabrook RW. Improvements in the isolation procedure for thyroid peroxidase: nature of the heme prosthetic group. *Arch Biochem Biophys* 1970;139:221–229.
15. Kaczur V, Vereb G, Molnar I, et al. Kinetic characterization of thyroid peroxidase enzyme and its inhibition by autoantibodies. *Acta Microbiol Immunol Hung* 1995;42:345–350.
16. Van Zyl JM, Van der Walt BJ. Solubilization of peroxidase from porcine thyroids and characterization by photoaffinity labelling. *Biochim Biophys Acta* 1984;787:174–182.
17. Danner DJ, Morrison M. Isolation of the thyroid peroxidase complex. *Biochim Biophys Acta* 1971;235:44–51.
18. Alexander NM. Purification of bovine thyroid peroxidase. *Endocrinology* 1977;100:1610–1620.
19. Taurog A, Dorris ML, Yokoyama N, et al. Purification and characterization of a large, tryptic fragment of human thyroid peroxidase with high catalytic activity. *Arch Biochem Biophys* 1990;278:333–341.
20. Nakashima T, Taurog A. Improved assay procedures for thyroid peroxidase; application to normal and adenomatous human thyroid tissue. *Clin Chim Acta* 1978;83:129–140.
21. Mariotti S, Pinchera A, Marcocci C, et al. Solubilization of human thyroid microsomal antigen. *J Clin Endocrinol Metab* 1979;48:207–212.
22. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–275.
23. Westermeier R. Blotting. In: Westermeier R, Gronau-Czybulka S, Habeck C, eds. *Elektrophorese-Praktikum*. Weinheim, Germany: Wiley-VCH, 1990;53–64.
24. Garfin D, Bers G. Basic aspects of protein blotting. In: Baldo BA, Tovey ER, eds. *Protein blotting: methodology, research and diagnostic applications*. Basel, Switzerland: Karger, 1989;5–42.
25. Castillo V, Casas G, Perez W. Autoimmune polyendocrinopathy in dogs. Association of hypothyroidism, Addison's disease and diabetes mellitus: five cases. *Revista Cientifica, Facultad de Ciencias Veterinarias, Universidad del Zulia* 1999;9:305–310.
26. Kotani T, Umeki K, Matsunaga S, et al. Detection of autoantibodies to thyroid peroxidase in autoimmune thyroid diseases by micro-ELISA and immunoblotting. *J Clin Endocrinol Metab* 1986;62:928–933.
27. Yokoyama N, Taurog A. Porcine thyroid peroxidase: relationship between the native enzyme and an active, highly purified tryptic fragment. *Mol Endocrinol* 1988;2:838–844.
28. Kotani T, Umeki K, Hirai K, et al. Experimental murine thyroiditis induced by porcine thyroid peroxidase and its transfer by the antigen-specific T cell line. *Clin Exp Immunol* 1990;80:11–18.
29. Kaczur V, Vereb G, Molnar I, et al. Effect of anti-thyroid peroxidase (TPO) antibodies on TPO activity measured by chemiluminescence assay. *Clin Chem* 1997;43:1392–1396.
30. Engler H, Riesen WF, Keller B. Diagnostic value of autoantibodies against microsomal thyroid peroxidase (anti-TPO) [German]. *Schweiz Med Wochenschr* 1992;122:1976–1980.
31. Bermann M, Magee M, Koenig RJ, et al. Differential autoantibody responses to thyroid peroxidase in patients with Graves' disease and Hashimoto's thyroiditis. *J Clin Endocrinol Metab* 1993;77:1098–1101.
32. McLachlan SM, Atherton MC, Nakajima Y, et al. Thyroid peroxidase and the induction of autoimmune thyroid disease. *Clin Exp Immunol* 1990;79:182–188.

Appendix

Comparison of serum concentrations of TSH and free T_4 in samples with positive and negative results for thyroid-specific aAbs (anti-Tg, anti-T_4, or anti-T_3 aAbs).

Variables	All samples	aAb positive	aAb negative
High TSH and low free T_4	153	106	47
High TSH and free T_4 within reference range	37	16	21
TSH within reference range and low free T_4	22	16	6
TSH and free T_4 within respective reference ranges	144	52	92
TSH within reference range and high free T_4	9	5	4
Total	**365**	**195**	**170**